EFFECT OF DIETARY ZINC LEVELS ON PHOTIC RETINOPATHY IN THE PIG

By

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The pig was used as a model to study the effects of light and dietary zinc on the retina. Animals were fed one of three experimental diets with low, normal and high zinc levels for one week prior to light exposure. Animals were then exposed to various schedules of intermittent or continuous high intensity light (630 foot candles) in a photic injury unit and then sacrificed 72 hours or 3 weeks post-exposure. The zinc status of animals was evaluated by several methods to allow observation of any influence of dietary zinc on light damage. Metallothionein in the neural retina was quantitated by the cadmium-hemoglobin binding assay.

Two types of light damage were induced in the porcine retina. The first type was characterized by multifocal outer nuclear layer pyknosis and outward movement through the external limiting membrane. The second type of damage was characterized by diffuse retinal edema, INL and ONL necrosis or swelling, and RPE damage. This more severe type of damage was rarely observed in the intermittent light group, was common in the continuous light group, and was noted in heat stressed animals exposed to intermittent light.

The light induced retinal damage was mild and asymmetric in many animals and at three weeks post-exposure recovery had occurred which suggests the light dose was near the threshold for photic injury. The experimental diets did not significantly alter the zinc status of animals so no conclusions can be drawn about the effect of zinc status on photic injury in the porcine retina. There was, however, a trend towards greater outer nuclear layer cell death in animals with lower kidney metallothionein, a good indicator of zinc status. Metallothionein levels were quantitated in the neural retina but were not significantly affected by dietary zinc level or by light-stress.

A low level of melanogenesis was observed in the RPE of unexposed eyes of young pigs. Low and high dietary zinc levels appeared to increase melanogenesis in the porcine RPE. Light stress resulted in an increased frequency of melanogenesis in the porcine RPE.

CHAPTER 1
INTRODUCTION

Excessive exposure to high intensity light results in pronounced damage to the retina. The cumulative effect of light exposure over many years is one factor thought to contribute to the development of age-related macular degeneration, the major cause of blindness in humans over fifty. The concentration of zinc in ocular tissues is higher than in any other tissue in the body, suggesting that zinc has important functions in the normal eye. A recent study investigating the effect of oral zinc administration on the visual acuity of human subjects with age-related macular degeneration revealed that individuals receiving zinc supplements demonstrated significantly less visual loss than non-treated individuals.[1] Using the pig eye as a model for the human eye, the proposed study will investigate the effect of zinc status on experimentally induced photic retinopathy.

The primary goals of this study are 1) to examine clinically and morphologically the damaging effects of excessive light exposure on the retina and choroid of the pig; 2) to examine by energy dispersive x-ray microanalysis any change in the elemental content of melanin granules in the retinal pigment epithelium and choroid that may occur during excessive light exposure; 3) to document the presence and distribution of the important zinc binding protein metallothionein in ocular tissues utilizing immunohistochemical staining and a cadmium-hemoglobin binding assay and 4) to examine and compare the influence

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of zinc supplementation and deficiency on photic injury, melanin granule elemental content and metallothionein levels.

Evidence exists which indicates that zinc can provide the retina cytoprotection during illumination. Exposure to excessive light has been shown to damage the outer layers of the retina and retinal pigment epithelium via disruption of their cell membranes.[2-4] The production of free radicals, H_2O_2, $OH^{\cdot}$ and O_2-, during excessive light exposure are key elements in the pathogenesis of photic retinopathy.[5-7] Zinc is found in high concentrations in the eye, especially the retina and choroid. Certain zinc-associated proteins including superoxide dismutase (SOD) and metallothionein are believed to play important roles as antioxidants in protecting tissues against free radical damage.[4,8,9] Superoxide dismutase is abundant in the retina and RPE.[10] A metallothionein-like protein was isolated in the bovine retina.[11] Subsequently, metallothionein has been found in human RPE cells and in the RPE and inner retinal layers of the rat.[12-19] In studies on the bovine retina, zinc had a non-uniform distribution being most concentrated in the outer segments which are very susceptible to lipid peroxidation during light and/or oxidative stress.[11] The protective role of zinc against photic injury is substantiated by another report that demonstrated zinc and taurine had a protective effect on peroxidation-induced damage of photoreceptor outer segments.[20] In these studies protection was believed to result from a membrane stabilizing effect.

Zinc may provide indirect protection against photic injury due to its essential role in maintaining the health of the RPE and retina. For example, retinol dehydrogenase is a zinc metalloenzyme that catalyzes the conversion of retinol to retinal which is used to recycle the photopigment rhodopsin.

The importance of zinc for normal retinal health is exemplified by the significant visual impairment and pathologic changes that can occur in ocular tissues of zinc-deficient animals and humans. Studies in boars fed diets deficient in zinc led to the development of a retinal degeneration characterized by dilation and disruption of the outer segments (especially of cone cells in the central retina) at the face of the retinal pigment epithelium.[21]

Since zinc is such a key element in the health of the retina, and zinc deficiency has been shown to induce retinal degeneration, it was hypothesized that zinc deficiency might increase the susceptibility of the retina to light-induced injury. Conversely, supplementation of zinc, an antioxidant nutrient, might protect against the oxidative damage that occurs in photic injury. Specifically, the hypotheses are as follows: 1) Dietary zinc supplementation has no protective effect on the degree of retinal damage produced by excessive light exposure, and the alternative, zinc supplementation is protective against photic injury to the retina, and 2) a zinc-deficient state does not increase the susceptibility of the retina to photic injury, and the alternative, a zinc-deficient state increases the susceptibility of the retina to photic injury. This investigation will examine the effect of altered zinc status on light induced retinal damage in order to provide a better understanding of how zinc and light may contribute to the retinal pathology seen in age-related macular degeneration.

The pig was chosen as an animal model due to important anatomical similarities with the human eye, including the presence of numerous cones that are concentrated centrally, the presence of red, blue and green sensitive cones, a similar retinal vascular pattern, and the absence of a tapetum lucidum that is present in most nonprimate mammalian species.[22-26]

CHAPTER 2
LITERATURE REVIEW

Photic Injury

Retinal damage due to excessive or prolonged light exposure is well documented and has been produced in the monkey, rat, mouse, rabbit, pigeon, dog, piglet, and man.[3,27-41] The degree and type of light damage to the retina depends on the type of stimulus used to produce the damage. Light damage may be assessed by light and electron microscopy, and by the electroretinogram. The most definitive and detailed morphologic picture of light damage is presented by studies with light and electron microscopy.[31,42,43]

Several factors influence the type of retinal damage induced by light. Variables that have been shown to influence light damage include body temperature, nutritional status, age, species, strain or breed, light intensity, duration of exposure, schedule of exposure (continuous vs. intermittent), light source (incandescent vs fluorescent), wavelength, and light history (cyclic, dark-adapted, etc.).[3,43] Because many variables influence the degree and type of damage, valid comparison between studies is difficult. The greatest amount of consistency is seen in the order and type of structural changes that occur in the retina.

Most photic injury studies have found that the first alterations occur in the outer segments(OS) of the photoreceptor cells. In rats, mice, monkeys and in piglets, rod OS appear swollen, lamellar structure is disrupted, and disc membranes thin and form tubules and vesicles.[3,30,31,35,39,44] Similar disruption of cone outer segments was observed in pigeons exposed to fluorescent light.[34] Pyknosis and swelling of mitochondria is then observed in the inner segment. Rod OS may become large, round or pear-shaped bodies. Microvilli of the RPE increase in length and number, and increased phagocytosis of the disrupted OS is evident. Outer segments lose connections with inner segments and begin to disappear. Inner segments shrink and pyknosis begins to occur in the outer nuclear layer. Ultimately, all damaged photoreceptors disappear. Kuwabara and Gorn commented that with extremely intense light or elevation of body temperature changes may occur in the cell body prior to outer segment disruption.[31] Although inner retinal layers usually remain normal in appearance, severe photic damage may result in degeneration of inner retinal layers. Müller cells proliferate to maintain retinal architecture. If photoreceptors completely disappear Müller cell processes and RPE microvilli interdigitate in a permanent adhesion.[43]

In Kuwabara and Gorn's studies in rats, birds and primates, the changes described above occurred over a one month period following exposure to fluorescent light. Similar changes have also been observed in primates [3,32,33,35,44], pigeons [34], piglets[30], and rabbits.[45]

The above descriptions represent the general sequence of changes that occur in photic injury, but as mentioned previously the changes seen can vary depending on the experimental conditions. Depending on the study, varying degrees of RPE damage have also been observed. For example, in some studies, incandescent light, very intense or long light exposure, and elevated body temperature resulted in more marked pigment epithelial damage.[3,39,44,46] It has been proposed that RPE damage is a result of thermal injury due to absorption of light and subsequent heat generation by melanin granules of the RPE.[47] Investigators also disagree on when RPE damage occurs. For example, in the rat photic injury model, Hansson believes damage occurs prior to receptor destruction, Noell believes it occurs simultaneously and Dowling believes it occurs subsequent to receptor destruction.[39,43,48]

Most studies have shown that with mild or moderate injury, retinal changes are reversible. Recovery begins in the RPE and proceeds very slowly with the formation of new lamellar membranes in the outer segments. Kuwabara believes the preservation of the pigment epithelium and the photoreceptor cell body are necessary for recovery. [32,43,48]

Mechanisms

Light may damage the retina in three ways: thermal, acoustic and photochemical.[2,49,50] In thermal damage significant temperature elevation is produced in the retina to cause coagulation of proteins and membranes leading to cellular damage. Acoustic damage is the result of ultrashort exposures to electric fields, acoustic signals or shock waves. Exposure results in production of sonic transients that mechanically disrupt tissues. In photochemical damage a molecular sensitizer is activated by incident photons which result in the production of free radicals. Free radicals are short-lived atoms or molecules that have unpaired electrons in their outer orbital rim. Because of this unstable structure they are reactive with other molecules and toxic to tissues.[38] Photochemical injury is usually produced with light intensities several orders of magnitude below that needed to produce a thermal burn and requires longer exposure times to produce damage, i.e., seconds, minutes or days vs. pico- or nano- seconds for the other mechanisms.[51] It is important to remember that there is no sharp line of demarcation between these three types of retinal injury.[3,49] Other proposed mechanisms of photic damage to the retina include metabolic disorders from extended overbleaching and production of toxic products other than free radicals in the outer layers of the retina.[52]

Photooxidation and lipid peroxidation. The mammalian retina is unique in that it is the only tissue where light is focused on a group of highly oxygenated cells. In the eye, blood flow per gram of tissue is higher than any other body tissue; for example, it is four times greater in the choroid than in the renal cortex.[42] The mammalian retina again is unique in its high content of polyunsaturated fatty acids (PUFAs). Polyunsaturated fatty acids are highly susceptible to lipid peroxidation induced by free radicals (esp. oxyradicals) and their susceptibility is directly proportional to the number of double bonds, i.e., the degree of unsaturation. The content and distribution of fatty acids in the retinas of humans, pigs, dogs, sheep, rabbits and cows are nearly identical.[53] Approximately 45% of the fatty acids in retinal membranes contain polyunsaturates and about thirty percent of the PUFA's in the retina is 22:6 docosahexaenoic acid (6 double bonds).[5,53] A reinvestigation of the PUFA content in frog, bovine and rat eyes found 46-50% 22:6 docosahexaenoic acid in rod outer segment membranes.[54] The combination of high oxygen tension and light exposure to the rod OS renders them particularly susceptible to light-induced oxidative damage (photooxidation). Photooxidation and lipid peroxidation are both believed to be significant contributors to photic retinopathy.[42,55]

In photooxidative damage a molecular sensitizer(S) absorbs a photon of light and is converted to an activated single state(^1S). The ^1S molecule is very unstable and short-lived and may dissipate its energy by fluorescence, interaction with the solvent or by radiationless transition to the triplet state(^3S).[4,5,42,56-58] The ^3S molecule mediates two types of photosensitized reactions. In type I the ^3S reacts with a substrate molecule, which may be another sensitizer molecule. In type II reactions the ^3S molecular reacts with oxygen to generate singlet oxygen or the superoxide ion radical $(0_2\cdot)$.[5,42] Singlet 0_2 reacts with water or other oxygen molecules and through univalent electron transfer can generate superoxide ion, hydroxyl radicals($OH\cdot$) and hydrogen peroxide. In addition, hydrogen peroxide can react with the superoxide radical or ferrous iron (Fenton's reaction) to produce the extremely reactive hydroxyl radical. These oxyradicals can directly damage tissues and especially can react with biomembranes to induce lipid peroxidation.

Lipid peroxidation is induced when a free radical ($R\cdot$), i.e., the hydroxyl radical, extracts a hydrogen and electron from a PUFA (LH_2) to form a lipid free radical.[5,56,58] This represents the initiation of the lipid peroxidation chain reaction:

$$RH\cdot + LH_2 = RH_2 + L\cdot$$

Propagation occurs when the lipid radical reacts with oxygen to produce lipid peroxide radicals ($LHOO\cdot$) and these peroxyradicals can react with other PUFA's to generate lipid hydroperoxides (LHOOH) and more lipid free radicals. The net effect is to oxidize lipids to hydroperoxides:

$$LH\cdot + O_2 = LHOO\cdot$$

$$LHOO\cdot + LH_2 = LHOOH + LH\cdot$$

Since more lipid radicals are generated that may react with other PUFA's, the cycle continues and is known as autoxidation.[4,57] Lipid hydroperoxides are capable of causing lipid peroxidations in vivo and in vitro by a chain reaction which may occur by fragmentation to an alkoxy radical and hydroxyl radical each of which continue chain reactions with additional unsaturated lipids.[56] The reactions above are only a few of many potential reactions that have the potential for enormous amplification of the initiating event which can be caused by any radical and could account for extensive lipid peroxidation initiated by very small amounts of oxygen derived radicals.

The lipid peroxide radical can decompose to form the highly reactive molecule malonaldehyde. Malonaldehyde is stable enough to diffuse to distant sites within the cell such as the nucleus.[57] Malonaldehyde readily condenses via Schiff base formation with nitrogenous materials including amino acids, peptides, phospholipids and nucleic acids. As a result, important functions of proteins (structural and enzymatic) and DNA (replication and transcription) are inhibited leading to cell damage and death.

Several investigators have provided in vivo and in vitro evidence that substantiates the occurrence of photooxidation and lipid peroxidation in retinas exposed to light. [6,42,59-61] Ham has shown a sharp drop in the radiant exposure threshold with increased partial pressure of oxygen in arterial blood in macaque monkeys exposed to blue light.[42] In that experiment pretreatment with methylprednisone (a membrane stabilizer) or beta carotene (an antioxidant and potent scavenger of singlet oxygen) protected against the oxygen potentiated damage. In another study, dark adapted frog retinas were shown to accumulate hydroperoxides when exposed to light.[6] The action spectrum of this process paralleled the absorption spectrum of rhodopsin having suggested this photopigment may participate in the induction of lipid peroxidation. Delmelle exposed retinal enriched liposomes to light and observed malonaldehyde production along with increased hydroperoxides, increased membrane fluidity and liposome lysis.[5,61] Porcine retinas irradiated with ultraviolet light had a significant increase in malonaldehyde, a product of lipid peroxidation of membranes.[62] Increase in hydroperoxides has also been shown in rabbit retinas exposed to damaging light.[59] Vitamin E deficient Wister rats exposed to high intensity light had three times the accumulation of hydroperoxides compared to controls indicating this antioxidant nutrient was protecting against lipid peroxidation mediated damage.[63]

In rats exposed to varied lighting conditions photoreceptor degeneration was accompanied by a selective loss of 22:6 docosahexaenoic acid and a simultaneous increase in hydroperoxides.[59,60] Since 22:6 is the major PUFA of rod outer segment membranes, its selective loss indicates lipid peroxidation of outer segment membranes may be a significant factor in photic retinopathy.

An important unanswered question is which molecule(s) in the retina represent sensitizers. Absorption of photons by sensitizers generates the first excited molecule that can participate in photooxidation and initiate lipid peroxidation. Potential sensitizers include exogenous substances (drugs, foods or dyes) and endogenous molecules. Rhodopsin, cone photopigments (i.e. iodopsin), flavin, melanin, retinal, retinol and the intermediates that occur in the light induced isomerization of rhodopsin (i.e. metarhodopsin I and II) are potential sensitizers.[2,5,37,42,64] Rhodopsin and cone photopigments are probable mediators of photodynamic reactions (directly or indirectly via their intermediates) as the action spectrum of light damage in the rat parallels the absorption spectrum of rhodopsin and in experiments utilizing narrow band filters selective cone damage has been produced.[2,29,43,55]

The above theories and experiments indicate that the retina is susceptible to photooxidative and lipid peroxidation mediated damage. Autoxidation in the RPE cell and phagocytosis of shed disc outer segments results in accumulation of the end products of lipid peroxidation, i.e., cross linked proteins, DNA and lipids in the RPE. The RPE enzymes, presented with an altered substrate, cannot always completely digest these materials and as a consequence residual bodies and lipofuscin accumulate in the RPE cells.[4,65] Lipid peroxidation probably occurs throughout an individual's lifespan. The age-related accumulation of lipofuscin in cells such as the RPE is ubiquitous across species and is thought to be important in the pathogenesis of ARMD.[4]

Other mechanisms. Several investigators have shown that the threshold for light damage decreases exponentially as a function of decreasing wavelength (i.e. increasing energy) indicating other mechanisms besides photopigment mediated reactions contribute to photic retinopathy.[40,41,43,49,52]

In rats exposed to continuous light, changes include activation of lysosomal enzymes and elevation of cathepsin D (total and free) in the neuroretinas, RPE and choroid when compared to control animals.[66] Lysosomal enzymes may contribute to photic retinopathy if excessive activation occurs under excessive light stress.

A recent study has shown that inactivation of cyclic GMP(cGMP) phosphodiesterase (PDE) accompanies light damage.[3] Cyclic GMP is a central molecule in phototransduction; the conversion of light energy to a nerve impulse. After light strikes a photoreceptor outer segment the photopigment (i.e. rhodopsin) is isomerized which induces a series of reactions that activate cGMP PDE. A subsequent decrease in cGMP results in closure of sodium channels and hyperpolarization of the photoreceptor and initiates an action potential.[67] In the Irish Setter and C$_3$H mouse an increase in cGMP secondary to a decrease in the PDE activity is thought to contribute to retinal degeneration. [68,69] The inactivation noted in light stressed retinas would result in an accumulation of cGMP that could contribute to the light induced retinal damage.

Other studies have shown inactivation of cytochrome oxidase occurs during exposure to ultraviolet light. [70,71] This mitochondrial enzyme is a component of the electron transport chain that is responsible for energy production for the photoreceptor. Photoreceptors have high energy demand to fuel the continual synthesis of dozens of outer segment discs that are shed daily. Inactivation of cytochrome oxidase during excessive light exposure could contribute to retinal damage.

In conclusion, multiple factors and mechanisms contribute to light mediated retinal damage. High oxygen tension, focusing of light on the retina, high PUFA content of outer segments and high metabolic rate of the photoreceptor make the retina very susceptible to damage mediated by photooxidation and lipid peroxidation. In addition, inactivation of important photoreceptor enzymes may contribute to light induced retinal damage.

Protective Adaptations in the Eye

It is evident the eye is susceptible to damage from excessive light exposure mediated by photooxidative and lipid peroxidative damage. Because the eye has evolved under these stresses a very effective set of physiologic and molecular adaptations have developed that protect the eye from light induced damage.

As mentioned previously the photoreceptors are extremely metabolically active. The ellipsoid region of the photoreceptor (PR) is packed with mitochondria to provide energy (ATP) for the cell. The primary activity fueled by the mitochondria is the constant renewal of outer segments. Both cones and rods continually synthesize outer segment discs, the discs move distally toward the RPE, and are eventually shed and phagocytized by the RPE.[71-77] Each retinal rod produces and sheds about 80-90 discs per day and the entire compliment of discs are replaced every 9 to 13 days.[76] The outer segments are very susceptible to photooxidative damage because of their high concentration of PUFA's. The constant shedding of older, more error-ridden parts of the PR outer segment allow for partial erasure of light induced molecular errors. Biological renewal, however, is not perfect, and the altered outer segments (i.e. with lipid peroxides) represent potential indigestible substrates for the RPE phagolysosomal system. Incomplete digestion of the shed outer segments results in accumulation of lipofuscin in the RPE and is part of normal senescence.[4,49,77] Excessive accumulation of lipofuscin can lead to pathology by further inhibition of RPE function.

Melanin granules in the iris, RPE and choroid represent another protective mechanism. One function of melanin is thought to be absorption of photons not absorbed by the outer segments and conversion of this scattered light to heat.[78] Recent investigations suggest melanin is a suppressor of photosensitized molecules including singlet oxygen, as well as a free radical scavenger.[49,78] In contrast, there is evidence that at high rates of exposure to short wavelength light melanin may become cytotoxic by production of free radicals.[49,64,79,80] This free radical generation is reversible and decay of the radicals occurs in the dark.[64] Melanin granules are in the apical region and microvilli of the RPE cells and the close proximity of these organelles to the rods and cones suggests they play an important role in the visual process. Some studies have shown pigmented animals are less susceptible to light damage.[2] The mechanism of this protection is related to iris pigmentation decreasing incident light on the retina. When retinal exposure intensity is identical, no difference in susceptibility of pigmented vs. albino rats was observed.[31]

In primates xanthophyll and other macular pigments are thought to be protective by absorbing the shorter and more damaging wavelengths of light. Some macular pigments may actually act as free radical scavengers.[49]

Many studies have shown that elevated body temperature lowers the threshold for retinal damage by light.[3,31,43] In addition to thermal mediated damage, chemical reactions that are facilitated by increased temperature may contribute to increased damage. As discussed above, the melanin granules in the RPE and choroid absorb excess photons and convert the energy to heat. The choroidal circulation is well designed to dissipate the heat generated by light absorption. The rich vascular plexus, the choriocapillaris, is separated from the RPE only by Bruch's membrane. Also, the larger choroidal vessels are arranged to result in a countercurrent flow pattern that aids in heat dissipation.[49] Recent studies have shown that choroidal blood flow increases with increased illumination.[81] The investigators believe the choroidal circulation, via regulation of blood flow, maintains normal body temperature in the RPE and outer retinal layers during light exposure and prevents excessive increases in temperature that predispose the retina to photic injury.

Several other intracellular mechanisms that protect against free radical and photochemical damage are present throughout the eye. Antioxidant nutrients such as alpha tocopherol (vitamin E) and ascorbic acid (vitamin C) are in high concentrations in the retina and other ocular structures.[49,82]

Many studies have shown vitamin C protects against light induced retinal damage.[82-85] In rats, guinea pigs and monkeys, intense light exposure leads to a loss of reduced ascorbate indicating it is oxidized during light exposure.[83] Administration of ascorbate before (but not after) light exposure led to a preservation of docosahexaenoic acid in rats having suggested this vitamin had inhibited oxidation of this major PUFA in rod outer segment membranes.

Vitamin E protects biological membranes from autoxidation and is present in high concentrations in the retina and RPE.[49,86,87] In experiments with light-induced retinal damage, vitamin E supplementation decreased retinal damage and decreased accumulation of hydroperoxides (end products of lipid peroxidation of membranes) when compared to controls.[63]

The RPE phagocytizes rod outer segments and with its active lysosomal system digests these membranous organelles. Incomplete digestion due to abnormal substrates (i.e., lipid peroxides) or decreased enzyme function results in formation of residual bodies or lipofuscin.[77,88,89] Accumulation of lipofuscin occurs normally with increasing age. Excessive accumulation of lipofuscin in the RPE may actually inhibit RPE function and result in retinal degeneration.[65,89] In rats and monkeys fed vitamin E deficient diets, massive amounts of lipofuscin had accumulated and severe retinal degeneration had occurred, both having been attributed to lipid peroxidation.[87,90-93]

Taurine, an amino acid, is another potential antioxidant nutrient in the eye. Taurine deficiency causes retinal degeneration in cats.[94] The mechanism by which taurine maintains photoreceptor structure and function is thought to be a membrane stabilizer. Evidence for this was found in studies that showed taurine prevented peroxidation-induced damage in isolated frog rod outer segments via membrane stabilization.[23,95] In studies with rats, the retinas of taurine-depleted animals were not more susceptible to damaging light intensities than controls.[96] Unlike primates, rats or pigs, taurine is an essential amino acid in cats and the dietary-induced retinopathy seen in cats is probably unique to this species.

Zinc is another nutrient that indirectly and directly provides protection against light induced oxidative damage to the retina as metalloenzymes (i.e., superoxide dismutase and catalase), metalloproteins (i.e., metallothionein) and/or by stabilizing membranes.

Specific antioxidant enzymes, superoxide dismutase (SOD), catalase and glutathione peroxidase, catalyze the enzymatic breakdown and detoxification of superoxide and hydrogen peroxide. SOD catalyzes the dismutation of the superoxide radical to hydrogen peroxide and oxygen. SOD is 200 times more concentrated in bovine and frog rod outer segments than the rest of the retina.[10,49] Thus, this potent antioxidant enzyme is present in the portion of the retina most susceptible to oxidative damage.

Catalase converts hydrogen peroxide to water and is found in microperoxisomes in the RPE.[97,98] Microperoxisomes are intracellular organelles found in cells involved with lipid metabolism and are thought to provide protection from hydrogen peroxide damage.[99] Microperoxisomes actually decrease in number in the RPE at the corresponding burst of outer segment disc shedding that occurs with the onset of light.[97] The investigators propose microperoxisomes fuse with RPE phagosomes and facilitate digestion. Microperoxisomes are probably an important component of the steady state equilibrium of disc shedding and RPE phagocytosis/digestion because they prevent accumulation of undigestible and damaging lipoperoxides (which result in lipofuscin formation) in the retina.[98]

Glutathione peroxidase is another antioxidant enzyme present in the retina.[4] In the presence of reduced glutathione it can detoxify lipohydroperoxides, hydrogen peroxides, nucleic acid peroxides and other radicals.[100] It also can recycle other antioxidants, i.e., by reducing oxidized ascorbic acid produced during high light exposure.

Zinc and the Eye

In 1869 zinc was found to be essential for growth in fungi[101], in 1926 for higher plants[102] and in 1934 essential for rats.[103] In 1939 carbonic anhydrase was proven to be a zinc metalloenzyme.[104] In 1955 parakeratosis in swine was found to be secondary to zinc deficiency.[105] Several other studies have shown the importance of zinc for the growth and maintenance of animals.[106-108] In 1963, Prasad and others documented the importance of zinc for human health.[109] Clinical manifestations of moderate to severe zinc deficiency include anorexia, growth failure, alopecia, thickening and hyperkeratinization of the epidermis, gonadal hypoplasia, diarrhea, mental disturbance, altered cell mediated immunity leading to recurrent infections, delayed wound healing, abnormal dark adaptation, hypoguesia, and if untreated, death.[110,111] These studies and many others continue to document the importance of zinc for normal health in animals and humans.

The importance of zinc to the eye was suspected, when it was determined the content of zinc in the eye is higher than that of most mammalian tissues. The highest concentration is in the choroid and retina followed by the ciliary body, iris, optic nerve, sclera, cornea and lens.[112,113] In addition, investigators have shown a rapid uptake of zinc by melanin containing ocular tissues in several species.[114,115] The copper content of the eye is also proportionately high compared to other tissues.[113,116] In 1939, Keilin and Mann showed zinc was an essential component of carbonic anhydrase.[104] This discovery provided unequivocal evidence for zinc's role in ocular metabolism and paved the way for further investigation into the biological role of zinc in the eye.

Zinc has diverse functions in metalloenzymes, immune competence, visual acuity, wound healing, stabilization of membranes, gene expression, DNA structure and in protein, carbohydrate and lipid metabolism. [9,106-108,117-121]

Zinc can have a structural, catalytic or regulatory role in metalloenzymes. At least 51 zinc metalloenzymes have been identified and examples in all six enzyme classes can be found.[9] In addition to carbonic anhydrase several other zinc metalloenzymes function in ocular tissues: retinal dehydrogenase, superoxide dismutase, leucine aminopeptidase, collagenase and alpha mannosidase have been identified in ocular tissues.[10,112,122,123] Carbonic anhydrase is important for aqueous humor production and is also present in the RPE[18]. Retinal dehydrogenase converts retinol (vitamin A) to retinal which is a component of the visual pigment rhodopsin. Superoxide dismutase is an important antioxidant enzyme found in high concentrations in the retina.[10] Leucine aminopeptidase is utilized for the degradation of lens proteins. Collagenase is important in corneal wound healing.[112] Alpha mannosidase is a lysosomal enzyme in the RPE participating in the digestion of shed rod and cone outer segments.[122] From these examples it is obvious that alterations in zinc status could significantly influence metabolism in the eye and vision.

In all tissues, zinc in metalloenzymes that participate in nucleic acid and protein synthesis, is important in cell differentiation and proliferation. For example, in human RPE cell cultures, decreased zinc resulted in decreased cell proliferation (63%) and protein synthesis (50%).[14] In low zinc medium other parameters were also reduced in the RPE cells: zinc (40%), catalase (68%), alkaline phosphatase (60%), alpha manosidase (68%), and metallothionein (82%). After zinc repletion catalase and alpha mannosidase activities rose by 86% and 106%, respectively, suggesting inactive enzymes were present but were not functioning due to lack of zinc.[124] Another study on human donor eyes determined the amount of zinc in the peripheral and macular retina. Cytosolic zinc was higher in the peripheral retina vs the macula.[125] A positive correlation between macular and peripheral zinc, and between age and disease was found. A negative correlation between macular zinc and age, and macular zinc and disease was observed. For example, total zinc was 38% less in eyes of people over 70 compared to those under 70 years of age.[125] It is possible that age related decline in enzymes such as catalase, important proteins like metallothionein and other human RPE functions can be influenced by the amount of bioavailable zinc at the cellular level.[15] This reduction in the function of the RPE with age is an important component in the pathogenesis of ARMD.

Zinc may also provide protection against light-induced free radical damage via its role in promoting membrane stability. It is increasingly evident that zinc ions are an integral part of membranes.[119,120] A substantial amount of zinc is located in the plasma membrane bound to lipoproteins and other membrane-associated proteins, i.e., enzymes and structural proteins.[121] Elevated extracellular zinc leads to increased membrane zinc with a stabilization effect, and, conversely, decreased extracellular zinc leads to loss of plasma membrane-associated zinc and destabilization.[119] Chavpil and co-workers believe membrane pathology is a primary component of zinc deficiency pathology.[121] One of the mechanisms by which zinc promotes membrane stability is via prevention of lipid peroxidation.

Several experiments have shown zinc deficiency increases lipid peroxidation in membranes and that zinc protects against peroxidative damage induced by heavy metals, carbon tetrachloride and high oxygen tension. [119,126-128] In zinc deficient rats, liver microsomes had increased lipid peroxidation in vitro and in vivo.[129,130] Liver mitochondria and microsomes from rats fed high levels of dietary zinc showed resistance to in vitro induced lipid peroxidation.[121] Dietary induced zinc deficiency in rats increases endogenous free radical production in lung microsomes.[131] Zinc aspartate protected mice against lethal effects of ionizing radiation.[126] The radioprotection was attributed to formation of zinc complexes with low or high molecular weight thiols (sulfur containing agents) within cells. Zinc is thought to prevent free radical formation and oxidation of protein sulfhydryl grouOps by complexing with sulfur molecules and protecting against radical induction.[119,126]

The effects of altered zinc metabolism on the eye and vision are rapidly becoming an area of interest for many researchers. Specifically, an association between zinc deficiency and the development of age-related macular degeneration has been proposed.[1] It is currently believed suboptimal zinc nutrition poses a problem for a substantial section of the United States population.[111] Children, the aged, and pregnant individuals are especially at risk.[102,107,111,132-134] Several studies as well as clinical diseases have shown zinc deficiency results in significant structural and physiological alterations within the eye.

In rats low maternal plasma and embryonic zinc concentrations during organogenesis caused aberrant morphogenesis of the optic vesicle and incomplete closure of the fetal fissure leading to anophthalmia or colobomatous microphthalmia.[117] Microphthalmia has also been seen in chicks from zinc deficient hens.[117] Because zinc is important in protein metabolism and has a beneficial effect on wound healing in vascular tissues,[107,108,110,112] it is thought to promote corneal healing. Collagenases which contribute to devastating melting corneal ulcers contain zinc. Treatment of melting ulcers with zinc chelators (i.e. EDTA) promotes healing.[112] These contrasts reflect the complexity of zinc metabolism within the eye. Zinc deficiency induces cataracts in fish[135] and some believe may play a role in the etiology of cataracts in humans; however, evidence to substantiate this is lacking.[112,136,137]

Several human diseases, including acrodermatitis enteropathica, alcoholism, malabsorption, sickle cell anemia and chronic renal, pancreatic or debilitating diseases, are associated with zinc deficiency. A common manifestation of zinc deficiency is abnormal dark adaptation (poor night vision).[111,112,137-139] The rod photoreceptors with the photopigment rhodopsin are responsible for night vision. Two mechanisms contribute to the abnormal dark adaptation observed in individuals that are deficient in zinc.

Retinal alcohol dehydrogenase converts retinol (vitamin A) to retinal which condenses with opsin to form rhodopsin. One mechanism by which zinc deficiency results in abnormal dark adaptation or night vision is felt to be from decreased activity of retinal alcohol dehydrogenase. In zinc deficient rats retinal and liver alcohol dehydrogenase activity were significantly lowered and conversion of retinol to retinal reduced. The retina was more sensitive to the lack of dietary zinc than the liver.[123] Dietary or pathological zinc deficiency causes a functional vitamin A deficiency at the level of the retina because retinol is not converted to retinal. Vitamin A deficiency is a well documented cause of night blindness. In alcoholic cirrhotics, zinc and vitamin A deficiency are common. Morrison et al. found that in some patients that were initially treated with vitamin A, only after addition of zinc did dark adaptation return to normal. They suggest the improvement seen in dark adaptation may be due to enhanced activity of previously depressed retinal alcohol dehydrogenase.[138]

Zinc deficiency may also influence vitamin A metabolism at the level of the liver by decreasing synthesis and/or release of retinol-binding protein (RBP) from the liver.[118,137-142] Vitamin A is bound to RBP and prealbumin for transport to the retina and other tissues.[140,143] Retinol binding protein levels in liver and plasma were found to be 50% and 25% of normal in zinc deficient rats.[123] In zinc deficiency, albumin levels are also low, which may contribute to inadequate transfer of vitamin A to the retina.[140,141]

Acrodermatitis enteropathica is a rare hereditary abnormality in zinc absorption characterized by dermatitis, chronic diarrhea, central nervous system abnormalities and impaired immune function with frequent infections. Several ocular manifestations have been reported. Ocular signs including conjunctivitis, canthal dermatitis, linear subepithelial corneal opacities, cataracts, optic atrophy, ciliary body atrophy, retinal degeneration and RPE depigmentation have been reported.[137] Gaze aversion and photophobia have also been reported and are suggestive of impaired cone vision.[118] Similarly, in alcoholic cirrhotics with zinc deficiency, a few reports of abnormal color vision and macular vision have been made, indicating cone vision may be affected by zinc deficiency.[118]

Silverstone and coworkers believe alterations in zinc and copper metabolism are involved in pigmentary retinopathies including retinitis pigmentosa and ARMD.[144] In a group of retinitis pigmentosa patients they documented low plasma zinc while in ARMD patients they found elevated plasma zinc and copper.[145] The elevation of plasma zinc and copper was postulated to be due to a constant release of these elements from the pigment in degenerating RPE and choroid occurring in ARMD.

In spite of Silverstone's findings, zinc deficiency has been proposed to be one of the etiologic factors in ARMD. [1,136,146] In a study by Newsome and coworkers of a group of 151 subjects, the zinc treated group had significantly less visual loss than the placebo group.[1] Using the technique of elemental analysis, Ulshafer found that zinc levels in degenerating RPE cells adjacent to drusen deposits (both of which occur in ARMD) were decreased.[147] It should be mentioned that Silverstone 's finding of increased plasma zinc does not rule out a subclinical zinc deficiency in patients as assessment of zinc status is difficult, and plasma values are a poor indicator of status.

Another possible way zinc deficiency may contribute to ARMD is via alpha mannosidase. In a recent study an age-dependent decrease in alpha mannosidase activity was found in RPE cell cultures and addition of zinc to the media increased activity 3-fold.[122] The same investigators found a decrease in alpha mannosidase activity in the serum of ARMD patients. Addition of zinc augmented serum enzyme activity 2-fold.[124] Since alpha mannosidase is required for the degradation of phagocytized outer segment membranes in the phagolysosomal system of the RPE, decrease in its activity could contribute to the accumulation of undigested outer segments as drusen and lipofuscin, both of which are associated with ARMD. Zinc status of donor patients was not assessed in this study.

Histological and ultrastructural studies of the effects of zinc deficiency on the retina are limited. The eyes of a male child with acrodermatitis enteropathica were obtained for histopathological exam.[137] Extensive degeneration of the RPE throughout the posterior pole was observed as well as atrophy of the inner and outer retinal layers. Interestingly, loss of rod and cone outer segments was most severe anterior to the equator. Extensive loss of the ganglion cell and nerve fiber layers was present. Complete atrophy of the optic nerve was also observed. The optic nerve atrophy was attributed to the severe nervous system involvement, several historical episodes of cardiopulmonary arrest and previous treatment with diodoquin, all of which could contribute to severe optic nerve atrophy. The above history coupled with repeated infections in the child may also have contributed to the retinal degeneration so it is difficult to attribute the retinal changes to zinc deficiency alone.

Leure-duPree et al. have shown several alterations in the retina of rats on zinc deficient diets or treated with zinc chelators.[142,148,149] They observed accumulation of osmiophilic inclusion bodies within the RPE as well as vesiculation and degeneration of the photoreceptor outer segments. The photoreceptor degeneration was usually in the areas where many inclusions had accumulated in RPE cells. The inclusions did not appear to be the products of phagocytosis, nor did they resemble lipofuscin granules or microperoxisomes. The inclusions were lipid in nature but otherwise the chemical composition has not been investigated. Inclusions were usually associated with mitochondria and smooth endoplasmic reticulum. Modified light regimes did not affect or initiate formation of the inclusions.

Samuelson and coworkers have investigated how zinc deficiency affects the retina in swine.[21,150,151] Because extensive literature is available on zinc deficiency in pigs and the zinc deficiency syndrome can be consistently reproduced, this species represents a good model for studying the effects of zinc deficiency in the eye. [141,152-155] In boars maintained on low zinc diets for 4 months, numerous cone nuclei and their inner segments were displaced towards the RPE. Occasional cones were observed in various stages of degeneration. The number of cone inner segments appeared distinctly fewer in those animals that had been on the low zinc diet for 4 - 12 months. In boars on the diet for 8 months disorientation of the outer segments were noted. Boars fed a control diet and penicillamine had similar retinal changes. Current studies of sows maintained on chronic low and moderately low zinc diets have shown similar disruption of outer nuclear layers and the development of large melanosomes in the choroid. Elemental analysis of RPE and melanin granules in these animals showed interesting alterations in zinc, copper and calcium including a decrease in zinc in RPE melanin granules vs controls.[21,150]

Metallothionein

Metallothionein (MT) is a low molecular weight cytosolic protein that selectively binds metal ions such as copper and zinc. This protein also has affinity for potentially toxic elements such as cadmium and mercury.[8,156,157] Some of the metallothionein's biological functions include protection against heavy metal toxicosis, and regulation of copper and zinc metabolism.[158-166] Metallothionein can donate its zinc ions to activate zinc metalloenzymes. It is involved in cell proliferation and differentiation most likely as a provider of zinc ions, since some enzymes responsible for synthesis of nucleic acids and proteins require zinc. Metallothionein may also play a role in maintenance of protective barriers since it has been found in the placenta, RPE (the blood- retinal barrier) and blood-brain barriers.[15,18]

Metallothionein can provide cytoprotection in several ways. It has been shown to be an efficient scavenger of free hydroxyl ions in vitro.[8,21] It may protect against potential damage caused by heavy metals, as well as other compounds involved in inflammation or physiologic stress. For example, dexamethasone, cAMP and interleukin-1 induce synthesis of MT and many of these molecules are important participants in the inflammatory responses or stress.[8,57,158,161] Induced MT may bind toxic metals or donate zinc ions for stabilization of membranes or important metalloenzymes like the antioxidant enzyme SOD. Ultraviolet light has also been reported to induce synthesis of MT.[8] The increase in MT protein results from increased transcription of MT genes.

A metallothionein-like protein has recently been identified in the bovine retina.[11] Subsequently, MT has been found in human and rat RPE, and in the inner retinal layers of the rat.[18] MT was also localized by immunohistochemistry in the corneal endothelium and epithelium, and in the lens epithelium.[18] In adult human RPE cell cultures mean MT levels were 18 +/- 2.2 μg MT / mg protein[13] in one study and 8 μg MT/ mg protein in another.[167] Studies on metallothionein in human RPE cell culture have revealed that MT (similar to the distribution of zinc) is present in higher levels in the macula (20 μg MT / mg protein) than in the peripheral retina (11 μg MT / mg protein). They also found that MT levels decrease with age, with a much greater decrease occurring in the macula vs. the periphery (i.e. from 28 μg MT / mg protein in the late 20's to 4 μg MT / mg protein in the late 80's.[14] The significant decrease in MT levels in the RPE that occurs with age may play a role in the pathogenesis of age-related macular degeneration and warrants further study.

Metallothionein synthesis is induced in RPE cultures by incubation with ZnCl, cadmium, dexamethasone, phagocytosis of latex beads or rod outer segments, 1,25 dihydroxy vitamin D_3 and paraquat (induces oxidative stress).[13,16] The induction of MT during oxidative stress (incubation with phorbol myristate acetate, lipopolysaccharide, ferrous sulfate, hydrogen peroxide, paraquat, and phagocytosis of rod outer segments) has been correlated with the activation of two nuclear regulatory proteins, NF-kB and Ap-1, again indicating the increase in MT is due to increased gene transcription.[16] Free radical scavengers like DMSO blocked the induction of MT that occurred with addition of hydrogen peroxide, again suggesting the oxidative stress caused the increase in MT synthesis.

Another experiment showed induction of MT after addition of ferric iron to human RPE cell cultures.[17] The 2-fold increase in MT was blocked by the free radical scavenger, N-acetyl cysteine (NAC) or cycloheximide. Ferric iron activated the DNA binding of NF-kB. Again NAC inhibited this activation. Phagocytosis of bovine rod outer segments induced MT and activated NF-kB, which were both blocked by the iron chelator desferroxamine during phagocytosis. The authors postulate that free iron may play a role in generation of an RPE stress response that can occur as a result of phagocytosis of shed photoreceptor outer segments.[17] This and other studies substantiate other data that suggests MT can be an acute phase reactant protein, which presumably plays a role in protecting the cell.

Excessive light exposure, which induces oxidative stress, might also induce MT. The proposed study in which MT will be identified in the porcine retina utilizing the cadmium-hemoglobin binding assay and immunohistochemical staining will attempt to document whether changes in levels or distribution of MT result from photic injury and/or manipulation of dietary zinc levels. Immunohistochemical staining has been utilized to localize MT in the kidney and liver of rats, and in the brain of humans.[168,169] Most of these studies utilized immunoperoxidase staining to localize MT.

Colloidal gold immunocytochemistry has been found to yield superior results for localization at the electron microscopic level.[170-172] With the silver intensification technique applied to colloidal gold stained tissues, specimens can also be examined at the light microscopic level.[173,174] Immunocytochemistry of the retina at the light microscopic level allows localization to the retinal layers but is not as useful for localization at the cellular level. Use of the colloidal gold technique will allow more detailed localization of MT so will be utilized in this study. Specimens will be examined at the light and electron microscopic level to try to determine where MT is distributed in retina and specifically in which cells. This will add to our knowledge of the biological function of zinc and metallothionein in ocular tissues.

Light, Zinc, and Age-related Macular Degeneration

Age-related macular degeneration (ARMD) in humans is the major cause of blindness in people over fifty.[4,65] This disease involves degeneration of the macula, a cone dense area of the human retina. Major features of ARMD include its central location, its association with age, disruption of retinal pigment epithelium (RPE) pigmentation, formation of drusen, destruction of Bruch's membrane, and basal laminar deposits of amorphous or granular material between the RPE cells and their basal lamina.[122] All the major signs of ARMD increase in prominence with advancing age, but only in some individuals do they progress to the stage of pathology. The RPE plays a prominent role in this macular disease. Throughout life photoreceptor discs are shed in association with the visual process. The RPE phagocytizes and degrades these membranes in conjunction with lysosomes. It is proposed that abnormal molecules gradually accumulate within the RPE due to imperfections in the cell's digestive mechanisms.[65] The residues of incomplete molecular degradation accumulate in the cell and increasingly interfere with normal metabolism which presumably leads to abnormal excretion of debris and drusen under the RPE cell. In support of this theory, a recent study has shown a significant age-dependent decrease in activity of alpha mannosidase in cultured RPE cells.[122] Furthermore, the researchers demonstrated a 2-fold increase in the enzyme's activity in aged RPE cell cultures with addition of zinc. Since alpha mannosidase is a lysosomal enzyme that normally functions in RPE lysosomal digestion of photoreceptor disc segments, a decrease in this enzyme's activity could lead to accumulation of undigested rod outer segments and drusen, both of which are associated with ARMD. These theories suggest that ARMD is an advanced stage of a deteriorative process that takes place in all eyes, and the progression of any of several factors may lead to pathology.

The etiology of ARMD is multifactorial. Some of the proposed factors that contribute to the development of pathology include genetics, light iris pigmentation, prolonged light exposure, and most recently, nutritional deficiencies.[18,65,167,175] A study on the effect of oral zinc administration and the visual acuity outcome in 151 subjects with drusen or macular degeneration showed that the treated group had significantly less visual loss than the placebo group after a follow-up of 12-24 months.[1] Using the pig as a model, this study will investigate the effects of zinc deficiency and supplementation on photic injury utilizing clinical, histopathological, immunohistochemical, and ultrastructural studies. The changes in elemental content of different components of the retina and choroid will also be observed utilizing energy dispersive X-ray microanalysis. The primary goals will be to determine if zinc supplementation is protective against photic injury and if zinc deficient states increase the susceptibility of the retina and RPE to photic injury.

CHAPTER 3
MATERIALS AND METHODS

Materials

Animals

Thirty-one six- to eight-week old lightly pigmented pigs were obtained from the University of Florida Swine Unit. Animals were obtained a minimum of 4 days prior to starting the experimental diet to allow acclimation to their new environment and recovery from the stress of transport. Animals from Part 2 of the study were treated with oral trimethoprim-sulfa suspension 30 mg/ kg P.O. q 24 h for the first 7 days of housing. When not in the experimental photic injury unit, animals were housed in the University of Florida College of Veterinary Medicine Metabolic building in air conditioned pens with free access to water. The front of the pens had galvanized chain link fencing with a door.

Photic Injury Unit and Lighting Conditions

The experimental design of the lighting conditions and photic injury unit was in part from the advice from Dr. William Dawson, Department of Ophthalmology, University of Florida College of Medicine, who has graciously agreed to advise us in this matter, and from a variety of past studies involved in photic injury.[30,48] The photic injury unit was circular with white formica on the internal walls. It was necessary to elevate the unit 4 inches off the floor to facilitate cleaning. A reflective white wall board was placed on the floor to help maximize light intensity. The unit was elevated five inches off the floor by legs to facilitate cleaning and air circulation. Incandescent tungsten-halogen light sources, 4 outdoor flood lights (Philips Lighting Co., Somereset, NJ), were suspended over the unit. These lights generate broad spectrum visible light possessing wavelengths from 400-900 nm. The relative power (radiant energy in µw/ 10 nm/ lumens) along the wavelength spectrum increases steadily being less than 100 for shorter wavelengths, i.e., less than 500nm, moderate (100-200) for intermediate wavelengths, i.e., 500-700nm, and greatest (200-300) for longer wavelengths of 700-900 nm. The incandescent bulbs also have a significant amount of radiant energy in the infrared (non-visible) spectrum with wavelengths up to 1500 nm wavelength.[1] The photic injury unit could house only two animals at one time under the guidelines of the University of Florida animal welfare committee necessitating staggering of times of exposure to the light in the unit. Therefore at the time of exposure pigs were 8 - 11 weeks old.

During the initial pilot study the temperature in the unit averaged 90-93°F and animals exhibited a tendency towards hyperthermia with rectal temperatures of 105-106°F compared to the normal range of 101.6-103.6°F.[176] Rectal temperatures of the animals prior to placement in the unit were around 103°F. Several studies have demonstrated that elevated body temperature (hyperthermia) accentuates photic injury.[3,43,49] To minimize the number of experimental variables that might influence photic injury in subsequent trials, a 100% acrylic infrared (heat) absorbing shield (K-S-H Inc., Olive Branch, MI) and fans were utilized to maintain the temperature in the unit at 80°F. This resulted in lower rectal temperatures of 102-103°F.

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[1] Incandescent Lamps, General Electric Co. 1977

The plastic shield, floor and walls of the unit were cleaned once or twice daily to remove accumulated dust in order to maintain maximum illumination within the unit. Light intensity was measured with a J16 digital photometer (Tektronix, Beaverton, OR). The photometer was placed in the center of the circular unit with the sensor directed at the wall of the unit. A measurement was obtained and then the photometer rotated 90° for the next measurement. This was repeated until twenty readings were obtained. Mean illuminance of experimental lighting (with the plastic shield in place) was 630.7 ft.-candles +/- 81.6. Control lighting had a mean luminance of 194.7 ft.-candles +/- 77.2 (n = 20). Control fluorescent lighting illuminated cages housing unexposed animals a generated a gradient of illumination from front (261 ft.-candles) to back (115 ft.-candles) of the cages. For comparison, normal indoor illumination, i.e., office lighting, ranges from 50-100 ft.-candles. Outdoor illumination on a clear sunny day is 10,000 ft.-candles, and in shade or on an overcast day may be around 1,000 ft.-candles or less.[49]

Diets

Diet analysis was performed by the University of Florida Animal Nutrition laboratory under Dr. Joel Brendamuhl's direction. Prior to the acquisition all animals were fed a corn-soybean meal based grower diet (the control diet in part 1) at the swine unit. One week prior to the time they were placed into the photic injury unit, the animals were placed on their designated experimental diet. The experimental diets were fed until the day before animal sacrifice (animals were fasted for 18-24 hours before sacrifice). The pigs were fed either a control diet (ZnN, 100 ppm Zn), zinc deficient diet (Zn-, 11 ppm Zn) or control diet that was supplemented with zinc sulfate powder to contain approximately 300 ppm Zn

(Zn+). The control diet with 100 ppm zinc met all the nutritional requirements for pigs of this age.[177] In the intermittent light stress group and their controls, the zinc deficient diet was a casein-sucrose based feed supplemented with mineral/ vitamin mix lacking zinc to end up with the low zinc level of 11ppm. The other diets were corn-soybean based (Table 1). In the studies utilizing continuous lighting, the experimental diets were all casein-sucrose based and had similar zinc levels; Zn- 11 ppm, ZnN 93 ppm and Zn+ 240 ppm (Table 2). Unfortunately the results of diet analysis were not available until after the experiment and it is apparent that the supplemented diet in part 2 of the experiment did not contain 300 ppm but only had 236 ppm of zinc. The average incoming weight of the animals was 20 kg. Animals were pair fed 1.5 kg feed per day which was within the range of the average expected feed intake for pigs of this age and size.[177] Animals kept alive for three weeks after light exposure had their feed intake level increased as body weight increased to 2 kg of feed per day. Animals were fed from plastic or stainless steel containers to avoid extra zinc ingestion. Animals being fed the zinc deficient diet were provided with distilled deionized water free choice. As mentioned previously, when not in the photic injury unit animals were in cement pens with galvanized fencing at the front. Galvanized metal contains zinc so pens that housed animals on zinc deficient diets had peg board stopped to the chain link to prevent the animals from chewing on the metal and obtaining exogenous zinc.

Table 1: Diet analysis from Part 1 of the experiment. The control and zinc supplemented diets were corn and soybean meal based. The zinc deficient diet was a casein and sucrose based diet. (NDF = non digestible fiber; ppm = parts per million or mg/kg; *determined on a dry matter basis, all others are on an as-fed basis)

	Zn Deficient	Control (Zn normal)	Zinc supplemented
	Casein-sucrose	Corn-soybean	Corn-soybean
Dry Matter %	95.34	89.74	89.24
Crude Protein %	17.90	18.06	18.02
Crude Fat %	4.99	2.00	1.99
Ash %	3.27	5.20	5.26
NDF %	3.06	11.40	2.51
Zinc ppm *	11.17	100.39	296.86
Copper ppm *	22.46	16.68	12.22
Calcium ppm *	7608	8359	5741

Table 2: Diet analysis for Part 2 of experiment. All diets were sucrose casein based.

	Zn Deficient	Control (Zn normal)	Zinc supplemented
Dry Matter %	91.12	90.82	91.06
Crude Protein %	18.46	18.44	17.85
Crude Fat %	5.78	6.98	5.85
Ash %	4.72	4.67	4.79
NDF %	4.02	3.9	4.27
Zinc ppm *	10.85	92.03	236.87
Copper ppm *	18.42	20.91	17.97
Calcium ppm *	7542	7573	7682

Methods

Photic Injury

Pilot study. Two young pigs were obtained from the University of Florida swine unit. They were fed the control diet and were placed in the unit for either 36 or 72 hours of intermittent light. These animals were sacrificed 72 hours after exposure and tissues were processed for light and electron microscopy (see details in next section). Rectal temperatures were taken every morning. Results of morphologic analysis of these tissues were used to guide subsequent experiments.

Part 1: Intermittent light stress. Eighteen animals were utilized for experiments with intermittent light stress. From each diet group (Zn-, Zn+ and ZnN) 2 pigs were exposed to one of three lighting conditions: control lighting, high intensity-short-exposure (18 hr light 6 hr dark for two consecutive days) and high intensity-chronic exposure (18 hr light and 6 hr dark for four consecutive days). Therefore animals received intermittent high intensity light for 36 or 72 hours as indicated in Table 3. Prior to and after photic injury the animals were exposed to control light intensity for 18 hours each day until sacrifice which was performed 72 hours and 3 weeks post-exposure.

Part 2: Continuous light stress. An additional group of thirteen animals was obtained after completion of the first study. Four animals were in each diet group (Zn-, Zn+ and ZnN). Experimental diets were started one week prior to light exposure. Due to constraints in expense of diet and board, only one animal, fed the control diet, was not exposed to light stress. All three diets were casein-sucrose based. Animals on the zinc deficient diet were kept in stainless steel cages and provided with deionized water delivered in plastic tubing

Table 3. Summary of experimental design for Part 1 utilizing intermittent light exposure. Six animals were in each diet group. Two animals of each group were exposed to the three lighting regimes. One animal in each diet group was sacrificed 72 hours or three weeks after light exposure. L1: control lighting, L2: 36h intermittent light stress, L3: 72h intermittent light stress.

DIET	TOTAL ANIMALS	Kill 72 hours	Kill 3 weeks
Control diet (100ppm Zn)	6 (2-L1; 2-L2; 2-L3)	3	3
Zn deficient (11ppm Zn)	6 (2-L1; 2-L2; 2-L3)	3	3
Zn supplemented (300ppm Zn)	6 (2-L1; 2-L2; 2-L3)	3	3

from plastic reservoirs. Animals in each group were exposed to 72 hours of continuous light (630.7 ft. candles) in the photic injury unit. Three of the four animals in each group were sacrificed by lethal injection 72 hours post light exposure and the fourth three weeks post-exposure. The experimental design is summarized in Table 4. One animal, on the control diet, was not exposed to light and was utilized as the control for this part of the experiment along with the control animals from part 1.

Table 4: Summary of experimental design for Part 2 utilizing 72 hours of continuous light exposure. Four animals were in each diet group and all received light. Another animal not exposed to light was fed the control diet for 10 days and served as the control for this experiment.

DIET	TOTAL ANIMALS	Kill 72 hours	Kill 3 weeks
Control diet (100ppm Zn)	4 (L4)	3	1
Zn deficient (11ppm Zn)	4 (L4)	3	1
Zn supplemented (300ppm Zn)	4 (L4)	3	1

Clinical Examinations

Initial physical examination (including weight), ophthalmic examination with fundus photography, and blood sample collection was performed during the 4-7 day acclimation period prior to starting the experimental diets. These procedures were repeated prior to sacrifice. For these procedures animals were anesthetized with xylazine (1mg/kg) and ketamine hydrochloride (10-20mg/kg) injected intramuscularly in the neck. Euthanasia was performed using sodium pentobarbital injected through an ear vein.

To determine if retinal damage induced in this experiment could be detected by electrodiagnostic testing with a Nicolet Compact Auditory unit (Nicolet Instrument Corp., Madison, WI), animals exposed to 72 hours of intermittent high intensity light (L3) and maintained for 3 weeks post exposure had electroretinograms (ERG) performed prior to light exposure and prior to their sacrifice. For ERG exams, animals were induced with the xylazine-ketamine combination and maintained under general anesthesia with halothane gas delivered via a mask. The pupils were dilated with 1% tropicamide. Ten flashes were signal averaged to generate the ERG wave-form. Light-adapted (photopic) ERGs were obtained and then the animal dark-adapted for 5 minutes. Low and high intensity dark-adapted (scotopic) ERGs were then obtained.

Blood was collected for determination of complete blood count, chemistry analysis, and serum zinc and copper prior to and at the end of the experiment. Frozen serum was stored so determination Zn and Cu levels by atomic absorption spectrometry could be performed on all samples simultaneously.

Tissue Collection and Processing

After euthanasia both globes were enucleated and transferred to the lab for immediate fixation. The anterior portion of the globe was removed at the ora serrata. Four wedges of freshly dissected tissue from the temporal portion of the retina were made (Figure 1). In part one of the experiment, the most superior wedge (wedge 1) was placed in 2.5% glutaraldehyde (360mOsm, pH 7.4) and subsequently processes for morphological examination. Wedge 2 was placed in 10% buffered neutral formalin (BNF); wedge 3 in 2.5% glutaraldehyde to be processed for immunohistochemical staining; and wedge 4 was slam frozen in liquid nitrogen. In the slam freezing procedure, the neural retina was gently

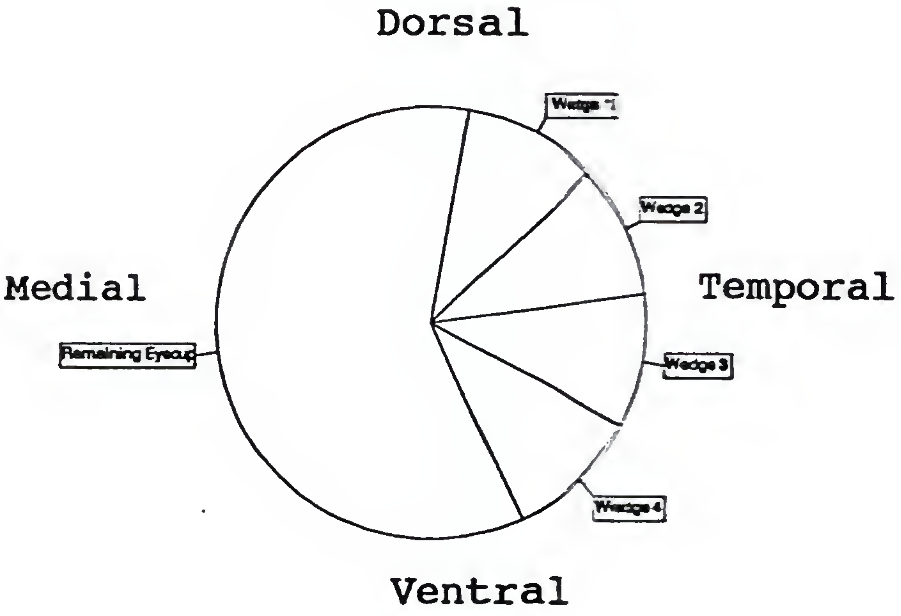


Figure 1: The pie portrays the posterior eyecup of a porcine eye after removal of the anterior segment. Four wedges were removed from each animal's eye and preserved by different fixation. The center of the pie represents the optic nerve. The area surrounding the junction of wedge 1 and 2 in the dorsolateral quadrant, represents the area centralis.

removed from the wedge and placed on a molybdemum (Mb) strip, grasped with pre-cooled copper block forceps and immersed in the liquid nitrogen. The rest of the wedge (with the RPE and choroid positioned to contact the copper block), was placed on a Mb strip and slam frozen as described above.

For eyes obtained from part 2 of the experiment wedge 1 was placed in 10% BNF, wedge 2 in combined 0.2% glutaraldehyde and 2.5% paraformaldehyde to be processed for immunohistochemical studies; wedge 3 was placed in 2.5% glutaraldehyde and processed for morphological studies; and wedge 4 was rapidly frozen by cold metal block freezing technique (slam freezing) with liquid nitrogen for x-ray microanalysis.

In morphological studies wedge 1 was used in part 1 (intermittent light stress and non-exposed controls) and wedge 3 in part two (continuous light stress) to enable detailed morphological examination of two different regions of the temporal retina utilizing both light and transmission electron microscopy. Wedge 1 includes the superotemporal retina including part of the area centralis and wedge 3 the inferotemporal retina.

After removal of the four wedges, the remaining neural retina was removed and frozen by immersion in liquid nitrogen. The underlying retinal pigment epithelium (RPE) and choroid were removed from the sclera and also frozen by immersion in liquid nitrogen. These tissues were stored for determination of metallothionein by the cadmium-hemoglobin binding assay.

Liver and kidney cortex were harvested from each animal. Tissues were fixed in the same fixatives described above and frozen by immersion in liquid nitrogen. Metallothionein is abundant in the liver and kidney cortex so sections of liver and kidney were used as positive controls for immunohistochemical studies. Frozen liver and kidney tissue were analyzed for copper and zinc content by atomic absorption spectrometry and for metallothionein content by the cadmium-hemoglobin binding assay.

Glutaraldehyde fixed tissues were left in fixative for at least 24 hours, washed in phosphate buffered saline, post-fixed in osmium tetroxide and dehydrated in a graded series of alcohols, alcohol propylene oxide mixtures and finally propylene oxide: plastic mixtures until embedding in 100% epon-araldite. Tissues for immunohistochemical techniques were processed without ossmication through a graded series of alcohol, alcohol-plastic and finally embedded in 100% LR-white plastic. For morphological and immunohistochemical studies, semithin (1 micron) and ultrathin (80-100nm) sections were made. One micron sections were stained with toluidine blue for light microscopic exam. Ultrathin sections were placed on copper grids for ultrastructural studies and post-stained in uranyl acetate and lead citrate.[170] Tissues fixed in buffered neutral formalin were routinely processed and embedded in paraffin. Five micron sections were cut and stained with hematoxylin and eosin for histological examination.

For energy dispersive x-ray microanalysis (EDX), following freezing, specimens were stored in liquid nitrogen until they were lyophilized by a freeze drying apparatus. The slam freezing technique is believed to be superior to other methods to allow x-ray analysis of biological tissues.[178,179] The specimens were then embedded in a low viscosity plastic resin (epon araldite) and 200-400nm semithin sections placed on formvar coated nylon grids (no post-staining).

Morphological Examination and Energy Dispersive X-ray Microanalysis

For the pilot study and part 1, a block of tissue from the center of wedge 1 (superotemporal), encompassing the area centralis, was mounted. For part 2 of the study, the similar central area of wedge 3 (inferionasal) was mounted and the adjacent mid-peripheral area was also mounted for sectioning with subsequent examination by light and electron microscopy. In part 2, selected eyes had central sections from the unosmicated wedge 2 mounted and examined as this wedge encompasses the area centralis. Electron microscopy was performed utilizing a Hitachi H-7000 transmission electron microscope.

Quantitative analysis of photic injury was attempted on tissues from animals in part 2 of the experiment. Light microscopic slides were placed on a microscope linked to an IBAS image analysis unit. The average of 25 measurements of retinal, ONL and RPE thickness were made on each eye. At 400x, an area was outlined and the total number of nuclei within the area was counted by placing a mark within each identifiable nucleus. This allowed determination of ONL density. After identifying the total number of nuclei in the outlined area, cone nuclei were identified to allow determination of the rod:cone ratio. This was performed by a computer program which subtracted the cones from the total number of nuclei in the area to determine the number of rods and then dividing the number of rods by the number of cones. Lastly, in the same outlined area, the number of degenerating/ dying nuclei were identified to allow calculation of the % dying cells. A nucleus was considered degenerating/dying if any of the following was present; pyknosis, significant alteration of chromatin pattern from normal, and displacement of the nuclei through the external limiting membrane (ELM). All of these counts were repeated five times on multiple sections from each eye. On the several sections from each eye, to facilitate statistical analysis, damage was also subjectively graded and the grades of damage were assigned numerical values as follows, no damage (0), mild damage (1), moderate damage (2), and severe damage (3).

For x-ray microanalysis the electron microscope was interfaced to a Kevex Super 8000 EDX unit. X-ray microanalysis is based on the principle that when atoms within an ultrathin specimen are struck by the electron microscope beam (i.e., electrons), x-ray waves of various energies (KeV) are emitted. Each element yields a group of x-rays that are characteristic of the atom (influenced by the element's atomic weight and valence for example) and thus the x-rays may be used to identify the elements present in the areas being scanned.[180] The EDX unit has a detector which collects the x-rays and displays them as a spectra with the number of counts per minute (relates to the amount of element in the area scanned) vs. the energy of the x-ray wave in KeV. Multiple x-ray emissions referred to as lines are generated as the electron beam strikes an atom and induces electron transitions with its electron orbits. For example, in the case of zinc the most intense lines, therefore most easily detected by the EDX, are the K-lines: $K_{\alpha 1}$ and $K_{\alpha 2}$ with energies of 8.638 and 8.615 KeV, respectively. The next most intense lines are the $K_{\beta}1$ with an x-ray energy of 9.572 KeV and the $L_{\alpha 1}$ line with an energy of 1.011 KeV. Several other less intense x-rays are also generated.[180] The less intense x-ray waves are not usually detected unless very high quantities of the element are present in the area being scanned.

Energy dispersive x-ray microanalysis was performed on tissues from animals in part 2 of the experiment, i.e., 72 hours of continuous light exposure. Elemental x-ray spectra were made by scanning a melanin granule with a preset time of 100 seconds. A minimum of five melanin granules were scanned from both retinal pigment epithelium and choroidal melanocytes from each eye. A background spectra of plastic without tissue was obtained from each specimen in which melanin granules were scanned to enable subtraction of any background elements.

Measurements were made on calcium, iron, copper and zinc peaks within each spectra. Several background measurements were made immediately adjacent to both sides of a peak and were averaged to determine the mean background height. The height of the peak was then measured and the peak/background (PK/BG) ratio determined (Figure 2). If any calcium, iron, copper, or zinc was present in the background spectra, the PK/BG ratio was determined similarly and then subtracted from the melanin granule spectra PK/BG ratios. Within an animal the PK/BG ratios of a minimum of five spectra were averaged for the RPE and the choroidal melanin. For the animals sacrificed 3 days post-exposure (3 in each group, therefore 6 eyes) the PK/BG values were averaged and statistical analysis performed.

Zinc and Copper Determination by Atomic Absorption Spectrometry

These analyses were performed on a Perkin Elmer 360 Atomic Absorption Spectrophotometer in Dr. Robert J. Cousins' lab, Department of Food Science and Human Nutrition, University of Florida. To determine zinc and copper concentration in tissues a nitric acid digest procedure followed by atomic absorption spectrometry (AAS) was utilized. AAS techniques are preferred because of their specificity, sensitivity, precision, simplicity and low cost per analysis.

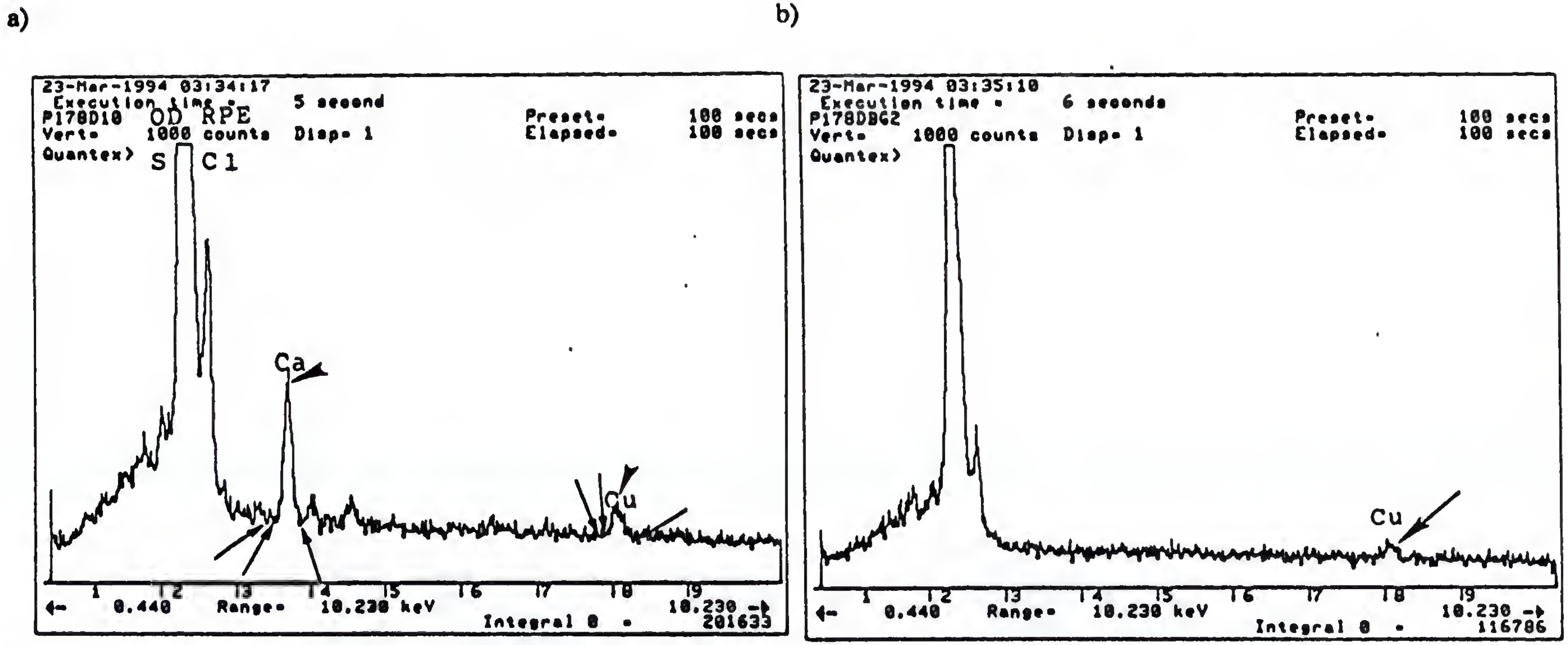


Figure 2: Typical elemental spectra of choroidal melanocyte melanin granule from a normal pig.

(a) Elemental spectra from RPE melanin granule. In (a) the arrows point to examples of where background measurements would be taken and the arrowhead points to the peak. The large peaks at the left end of the spectra are sulfur (S) and chloride (Cl) and were not routinely measured as these are ubiquitous elements in biological tissue and are also present in the embedding plastic. The peaks of interest in analysis of melanin granules are calcium (Ca), iron (Fe), copper (Cu) and zinc (Zn). In (b) the background spectra notably lacks Ca, Fe, and Zn. However a small amount of Cu (arrow) was typically found in background spectra and this was subtracted from the spectra obtained from melanin granules.

A standard curve was constructed using standards with 0.1, 0.5 and 1.0 ppm of the element of interest (copper or zinc). The absorbance of each standard is measured 3 or 4 times before and after measuring the unknown samples. Using the average absorbance of each standard and the known concentrations a linear regression was performed to construct a standard curve. The absorbance of the sample was then measured and the zinc or copper concentration calculated by interpolation from the standard curve. For zinc determinations the wavelength was set at 213.8nm and slit width 0.7nm. For copper determinations the wavelength was set at 324.8nm and slit width 0.7nm.

Serum samples. Whole blood collected by venipuncture in plastic syringes was centrifuged, the serum transferred into polyethylene storage vials and stored in a -30°F freezer until analysis. One milliliter of serum was diluted with 4ml of distilled water and mixed in a vortex. Distilled water was used as a blank. After calibration of the instrument and measurement of standards, the absorbance of each sample was measured.

Tissue samples. One tenth of a gram of tissue was placed in a 2.0 volumetric glass tube (wet weight). One milliliter of 75% nitric acid and 1 ml of 25% sulfuric acid is added to the tube and the tissues incubated in a hot bead bath overnight. After digestion of tissues 0.5ml of the solution is diluted 1:4 in distilled water in a separate glass tube and analyzed by AAS.

Immunohistochemistry

For immunocytochemical localization a gold labeled antibody technique was utilized.[171,172] Silver intensification was used on some sections for examination at the light microscopic level.[173,174] Tissues in Part 1 were fixed in 2.5% glutaraldehyde and in Part 2 in combined 0.2% glutaraldehyde-2.5% paraformaldehyde which is believed to provide better fixation for immunohistochemical staining.[170-172] After fixation, tissues were dehydrated in a graded series of alcohols, and embedded in LR white resin (Electron Microscopy Sciences, Fort Washington, PA). One micron and 80 nm sections were cut and stained followed by light and electron microscopic examination respectively.

For these studies, Dr. Mark Richards (USDA Agricultural Research Service, Beltsville, MD) generously provided approximately 10 micrograms of lyophilized porcine metallothionein I and II that were purified by high performance liquid chromatography. The metallothionein fractions were divided into three portions for antibody production in New Zealand White rabbits. The rabbits were bled seven days prior to their first immunization. Antigen was mixed with 0.5ml complete Freunds adjuvant and emulsified. Injection route was subcutaneous, intradermal and intramuscular. Thirty days after the initial immunization an antigen boost was given. The antigen was mixed with 0.5ml of incomplete Freunds adjuvant and emulsified and injected by subcutaneous, intradermal and intramuscular route. Ten days after the second injection a test sample was drawn.

The rabbit serum provided was tested utilizing an ELISA technique with a goat anti-rabbit IgG alkaline phosphatase conjugated antibody and also tested on sections of liver using a gold conjugated goat anti-rabbit IgG antibody. Dilutions used were 1:200, 1:400, 1:600, 1:800 and 1:1000 to determine if antibody against the porcine MT had been produced and which dilution was optimal for the immunohistochemical studies. A third antigen boost was given 14 days following the second boost. Two weeks later, animals were bled out and the serum used in immunohistochemical studies.

For colloidal gold staining the Auroprope LM and IntenSE M kit (Janssen Biotech, Olen, Belgium) kit was utilized. The manufacturers recommendations were followed with some modifications of the technique. For studies at the light microscopic level one micron sections were placed on clean slides. The staining procedure was as follows. First a 20 µl drop of 2% normal goat serum, a blocking agent, was placed on the sections for 1 hour at room temperature. The blocking agent is then removed by suction or blotting. The second step involves application of a 20µl drop of the primary antibody (the rabbit anti-pig MT antibody) at various dilutions. The ideal dilution was determined by performing several trials. The sections are incubated with the primary antibody overnight (usually around 16 hours) in a moist chamber at 4°C. The sections are then rinsed several times in phosphate buffered saline (PBS). The secondary antibody was a goat anti-rabbit IgG gold labeled antibody with 20 nm colloidal gold particles. The sections are incubated with a 20 µl drop of a 1:20 dilution of the secondary antibody for 8-12 hours at 4°C in a moist chamber. The ideal length of time of the incubation was determined by performing several trials. The sections were rinsed in phosphate buffered saline, a final wash in distilled water and then silver intensification performed. Silver intensification was done as recommended by the manufacturer.

For immunogold staining of ultrathin sections to be evaluated by electron microscopy silver intensification is not needed and the method was modified for use with ultrathin sections on grids. The same antibody dilutions and incubation times were utilized. Grids are floated on drops of antibody, PBS or distilled water instead of drops being applied to sections on slides.

Metallothionein Assay

In this study, metallothionein (MT) levels were determined in the pig utilizing the cadmium (Cd) hemoglobin (Hb) binding assay.[181] The assay was performed on neural retina, liver and kidney samples to add determine zinc status and any possible effects of diet and/or light. The method consisted of: 1) weigh the sample, dilute (usually 1:5) with TRIS buffer and homogenize (at this point, two 10μl aliquots of homogenate are removed for protein determination by the Lowry method); 2) centrifuge to obtain a supernatant fraction; 3) heat-denature the supernatant fraction, if high levels of MT are anticipated which would be out of the accurate range of the assay, dilutions of the supernatant with TRIS buffer can be performed; 4) incubation of 200 μl the supernatant fraction for 10 minutes with a 200 μl of a solution that contains 2.0 μg/ ml of cadmium total and 0.5 μCi of ^{109}Cd; 5) addition of 100 μl 2% bovine hemoglobin (Hb) solution; 6) heat precipitation of Hb; 7) centrifuge; 8) repeat steps 5,6 and 7; and 9) analysis of 100μl aliquot of the supernatant fraction for ^{109}Cd remaining in the fraction utilizing a gamma counter. In this assay ^{109}Cd remains in the supernatant fraction only in those samples containing MT. With this assay the amount of MT is calculated as follows:

$$\frac{\text{CPM (s)} - \text{CPM (bkg)}}{\text{CPM (t)}} \text{ X } (16.52) \text{ X initial DF X supernatant DF} = \mu\text{g MT/ mg sample}$$

The value of 16.52 is a constant determined by expressing the concentration of metallothionein in the solution and was obtained by dividing the grams of cadmium in each sample (for 200µl sample and 2 µg/ml Cd = 0.4µg) by the atomic weight of Cd (0.1124 µg/nmol) and the MT sample size (0.2 ml) to yield units of nanomoles of Cd/ml of sample. This value was multiplied by the molecular weight of MT (6500 Daltons) and then divided by 7, the number of Cd atoms that is bound by each molecule of MT. It is stated in the original paper that the accuracy of the assay is best if the sample counts are between 10 and 50% of the total counts. Therefore additional dilution or less dilution of the tissue in step 1 or the supernatant in step 2 was performed as needed if the sample counts were out of this 10-50% range.

To avoid variability produced by different wet weights of tissues the final MT value from the assay in µg/mg tissue is divided by the protein concentration (mg protein/mg tissue) determined by the Lowry method. This results in a value of µg of MT/ mg of protein.

Eyes from normal pigs at a University of Florida Meats Laboratory were utilized to test the assay on ocular tissues. Due to the small amount of ocular tissue (0.06 - 0.15 grams) modifications of the assay were required. The sensitivity of the assay was increased by increasing the amount of radioactive cadmium in the solution. Several runs were performed utilizing two test solutions: 1) 0.2 µg Cd total with 0.5 µCi Cd^{109}/ ml and 2) 1.0 µg Cd total with 1 µCi Cd^{109}/ ml. The choroid-RPE tissue was very scant, i.e., usually 0.05 grams, and also it was difficult to homogenize due to its high collagen content. The collagenous tissue tended to become caught in the homogenizer blades resulting in a loss of some of the already very small quantity of tissue. An ultrasonic homogenizer did not successfully homogenize the collagenous choroidal tissue without generating unacceptably high temperatures that would denature protein. All these factors made it impossible to accurately determine MT levels in the choroidal tissue. Several test runs of the assay did not result in accurate measurement of MT in the choroid-RPE tissues. In all trials the sample counts were much less (i.e., 4-6% of total counts) than the recommended minimum of 10% of the total counts which probably results in an underestimation of the amount of MT level.[181] Therefore, this assay was not performed on the choroid-RPE of experimental animals.

For neural retinal tissue, the solution with 1.0 μg Cd total with 1 μCi Cd^{109}/ ml gave consistent results in several test runs, so this solution was utilized on tissues from experimental animals. In addition to utilizing this solution in step 3, in step 1 all tissue was weighed and 0.35 ml of TRIS added (from the weight and the volume of TRIS the initial dilution factor was calculated). Because a different cadmium solution was utilized, the constant, which is based on the amount of total cadmium in solution, was recalculated and for the 1 μg Cd/ ml solution was 9.20. Because the amount of neural retina was small the tissue from both eyes of each animal was combined so only a single eye MT value was available from each animal.

Statistical Methods

Statistical analysis was not attempted on data from part 1 of the experiment because only one animal was present at each data point. In part 2 of the experiment, 3 animals comprised each diet group exposed to light and sacrificed 72 hours post-exposure, which enabled some statistical analysis. However, only one control (unexposed animal) per diet group limited the power of statistical tests.

Most variables of interest were observed in each of 2 individual eyes from 13 pairs of eyes belonging to 13 pigs. These pigs belonged to 1 of 3 possible zinc diet groups (4 in the Zn-, 5 in ZnN and 4 in Zn+ group). Within each of these diet groups, three pigs were exposed to 72 hours of continuous excessive photic conditions intended to induce retinal light damage, while the remaining pig in each group (the ZnN group had two non-exposed controls) remained unexposed to excessive light and were considered diet group-specific controls in the design. Single kidney and eye MT (combined tissues from both eyes) were determined from 9 additional pigs exposed to intermittent excessive photic conditions (2 fed the Zn- diet, 4 fed the ZnN diet [pilot study animals included] and 2 fed the Zn+ diet). This allowed comparison of kidney and eye MT values in non-exposed, intermittent and continuous exposed groups irrespective of diet, i.e., to see the influence of light on MT levels. For kidney and MT levels this resulted in an overall 2 factor independent groups design, with the zinc diet group factor having three levels and the light exposure factor also having three levels.

For response variables in which pairs of observations were available from 13 pigs, Pearson's correlation coefficient and Spearman's rank correlation coefficient were computed for OD vs. OS observations within subject to determine the degree of independence of consistency between eyes within animals. A paired T-test and the non-parametrically equivalent Wilcoxon signed rank test was also performed on the mean pairwise difference between eyes to determine if OD differed significantly from OS in any response variable. Paired observations within each pig were also averaged for all response variables except the subjective retinal damage scales (0-3, normal to severe) to obtain per-animal values for each pig. The maximum score within a pair of eyes was used as the per-animal values for each pig. All analyses were carried out separately on both individual eye datasets and per-animal data sets.

For all continuous variables other than kidney and eye MT, a completely crossed 2-factor independent groups analysis of variance (ANOVA) with 2 levels of light exposure (none or continuous) and 3 levels of zinc diet groups (Zn-, ZnN and Zn+) was used to determine statistically significant differences among various group means. These same variables were also analyzed in an analysis of covariance (ANCOVA) model in which kidney MT levels were considered as an indicator of dietary zinc status which could be substituted for the zinc diet group factor and used to adjust the light exposure group means before statistical comparison.

The outcome variable "percent cells dead" ranged from 0-100% and was therefore transformed prior to analysis using the arcsine-square root transformation in order to stabilize variance as a function of mean levels. Kidney and eye MT levels were also analyzed in a similar manner as described above except that an additional level for the light exposure factor categorizing MT levels in animals exposed to intermittent light was included in the 2 factor ANOVAs. One way ANOVA and the non-parametrically equivalent Kruskal-Wallis (KW) test were used to compare exposure group means within diet group and to compare diet group means within exposure group. The subjective retinal damage scale observations were only compared using the KW test. One way ANOVA and the KW test were then used to compare damage scale group means to see if continuous response variables levels were associated with the degree of subjectively assessed retinal damage.

Finally, all response variables in both the individual eye and individual animal datasets were correlated with one another using the Pearson and Spearman rank correlation coefficients. Pearson correlation coefficients were determined for elemental spectra (Ca, Cu, Fe, and Zn), percent dying cells, kidney MT, eye MT, ONL density and subjective damage. Only significant correlations will be presented. When the r^2 is greater than 30% it suggests the correlation may be real i.e. the % of variability of one variable is explained by the other variable.

CHAPTER 4
RESULTS

Animals

For Part I of the experiment, the facility utilized to house the animals being fed the zinc deficient diets was difficult to adapt to insure no exposure to exogenous zinc. The cages consisted of three sides of cement block and the front a section of galvanized chain link fence with a door. This section of galvanized metal was considered to be a potential source of zinc so a piece of peg board was secured to the fence to prevent the pigs from chewing on the metal. Deionized water was provided to all animals on the zinc deficient diets. The pigs chewed away sections of the pegboard and/or pulled the peg board off to expose the metal on several occasions. Thus they could have obtained exogenous zinc. To remedy this problem, in part two of the experiment, stainless steel cages were obtained to house the pigs fed the zinc deficient diets.

Fundus Photography

The normal fundus of the porcine eye is shown in Figures 3 and 4. The retina of the pig was holangiotic and atapetal. The optic nerve was well myelinated and horizontally oval with retinal arteries and veins on its surface (Figure 3). There was three or four large veins and 5-6 smaller arterioles. Dorsotemporally the vessels arced around a grossly avascular area centralis (Figure 4). None of the experimental animals fundic examinations revealed

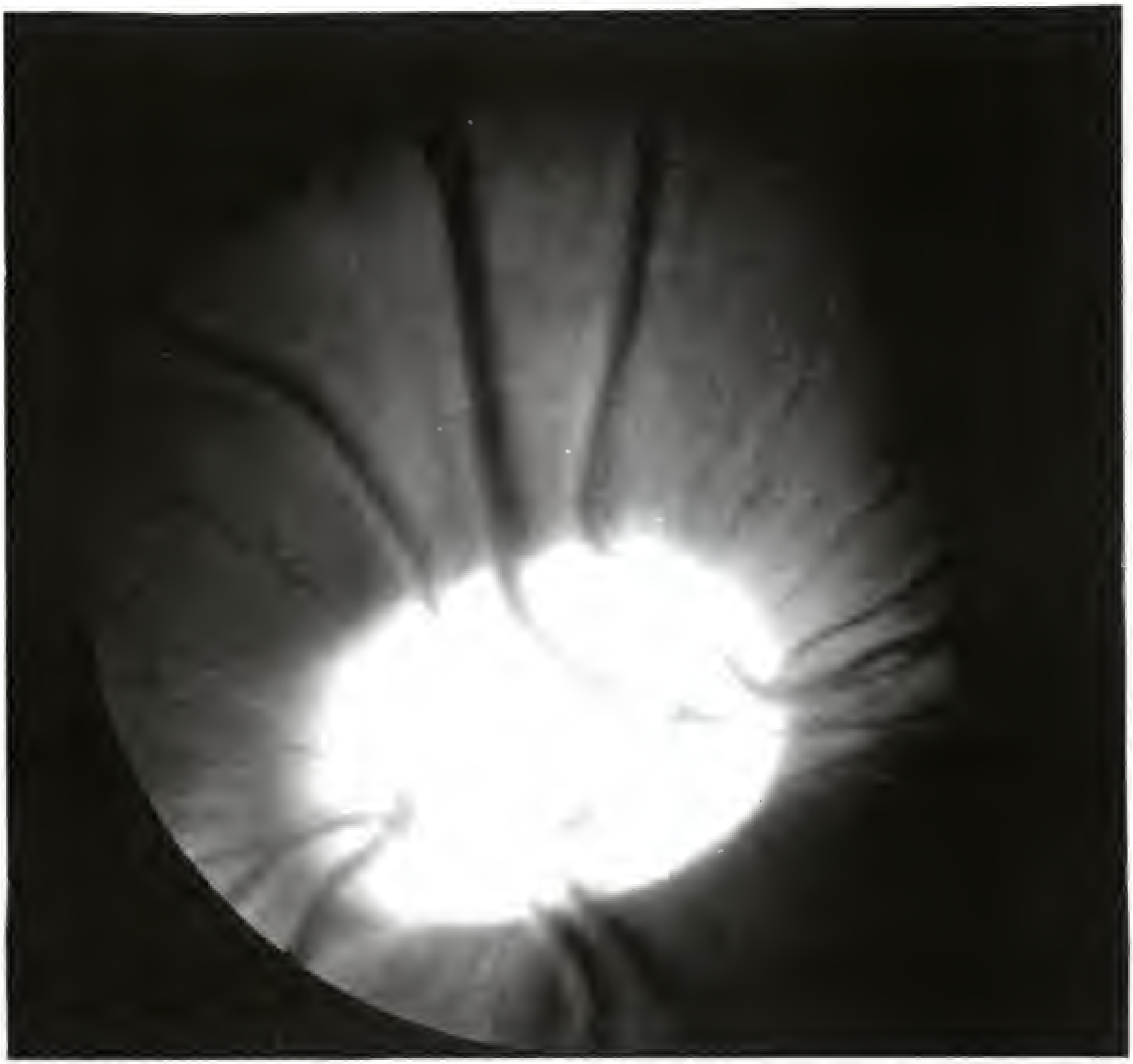


Figure 3: Normal porcine fundus. The area of the optic nerve is shown. The holangiotic vascular pattern is evident by the numerous vessels on the surface of the optic nerve. The porcine retina however, does not possess a central retinal aretery as is present in humans.

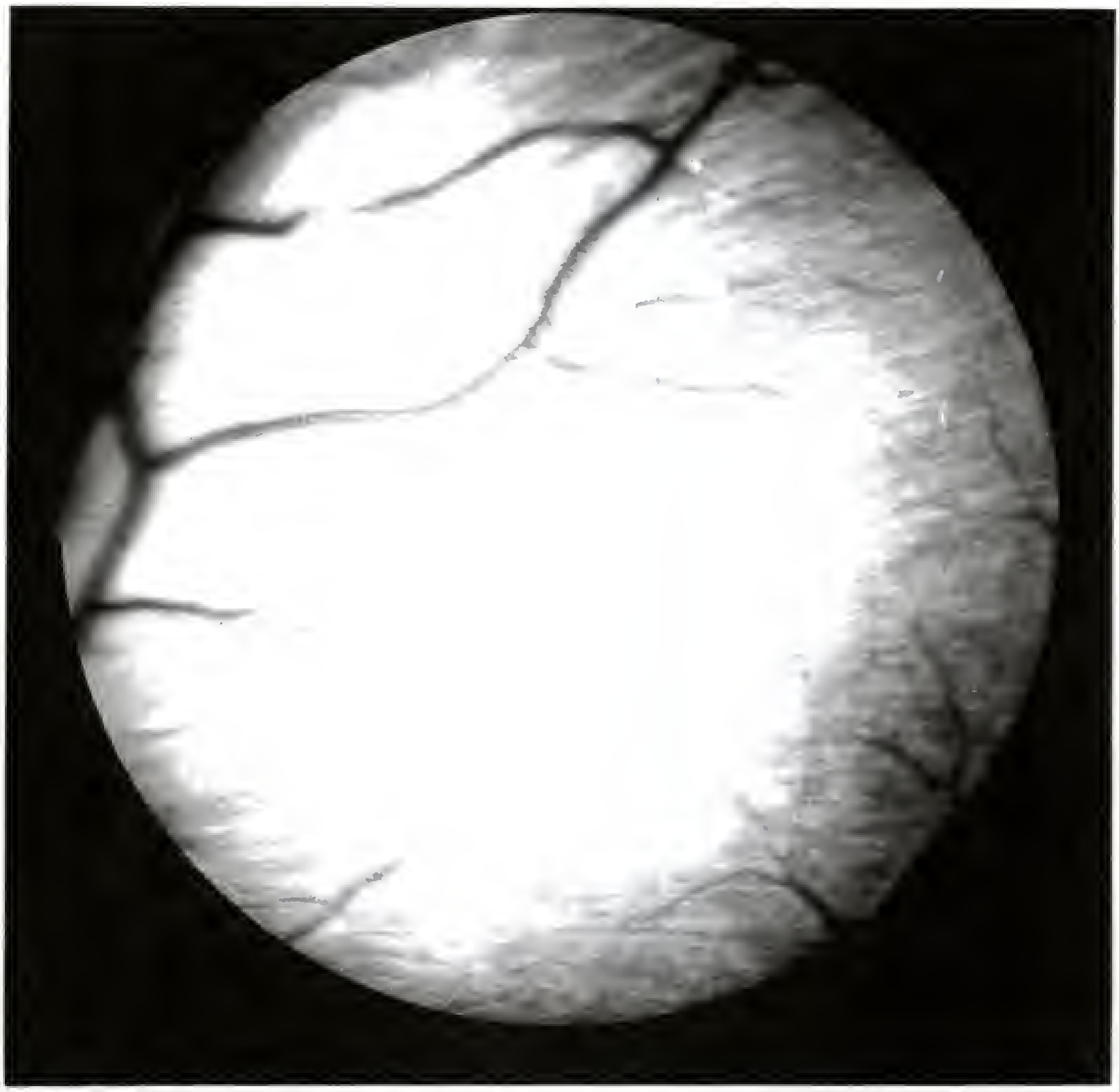


Figure 4: Normal porcine fundus with area centralis shown. Note vessels arcing around this dorsotemporal area to create a relatively avascular area.

clinically apparent retinal damage at 72 hours or 3 weeks post-exposure irrespective of diet or light treatment.

Electrophysiologic Studies

Electroretinograms were performed on selected animals from part 1 of the study prior to light exposure and then three weeks after exposure prior to euthanasia. The typical electroretinogram obtained from all pigs is shown (Figure 5). The amplitude of the b-wave actually increased during the three weeks between the initial pre-exposure evaluation and the repeat test 3 weeks after 72 hours of light exposure. No difference in b-wave amplitude or latency were noted in pre and post-exposure electroretinograms of the animals fed the Zn-, ZnN or Zn+ diets for three week periods after exposure to 72 hours of intermittent light. Because no changes were found in the intermittent light group, ERG testing was not performed on the continuous light group.

Blood and Tissue Analyses

Complete blood count and chemistry analysis performed on all animals at the beginning of the experiment revealed no abnormalities. Values were within normal limits of young pigs for the clinical pathology laboratory at the University of Florida College of Veterinary Medicine. In the control group fed the diet for 11-13 days there was one animal fed the Zn- diet and one fed the Zn+ diet. Two animals were fed the ZnN diet so the average of the two values, i.e. serum, kidney MT, were utilized in statistical analysis.

Serum samples drawn at the beginning and end of the experiment (at sacrifice) were analyzed for copper, zinc and calcium. Results are shown in Tables 5-7. Statistical analysis revealed no significant change in serum levels of copper or zinc as a result of feeding the

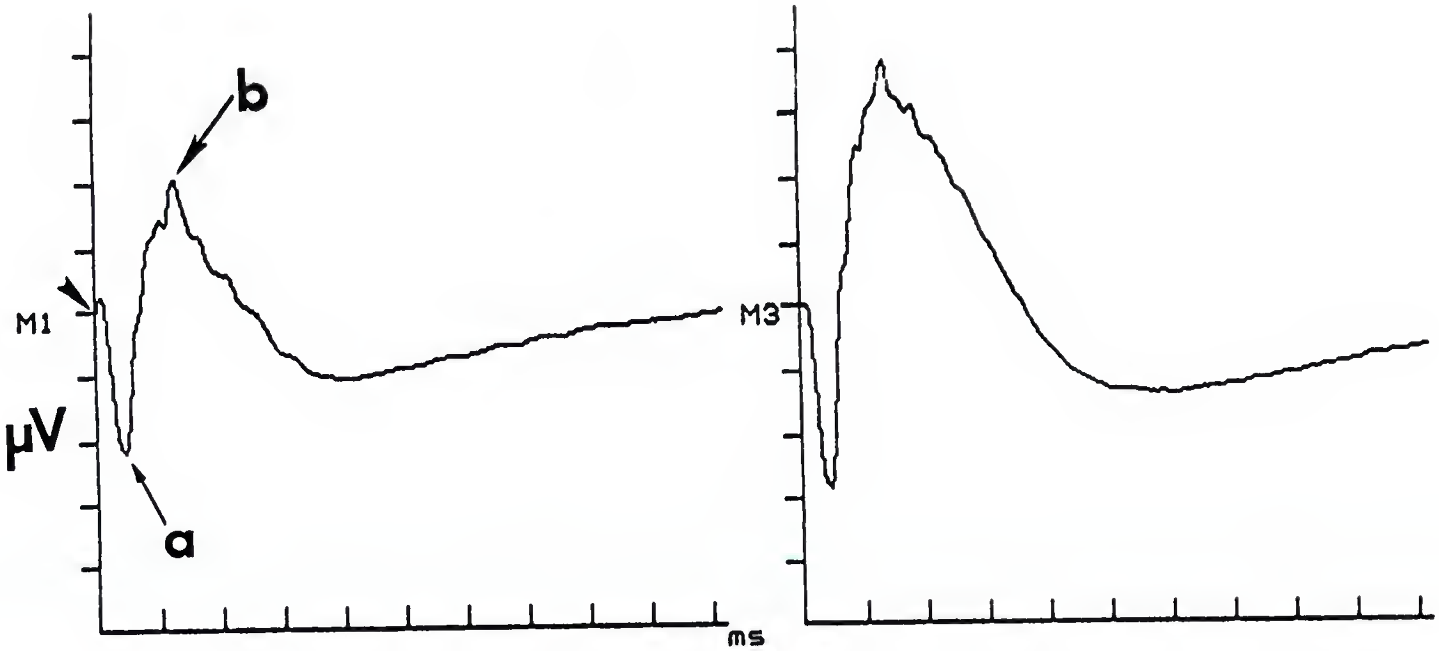


Figure 5: Normal electroretinogram (ERG) from young porcine eye. Electroretinography was performed on 3 animals from part 1 of the study and revealed no decrease in b-wave amplitude as would be expected if significant light damage had occurred.. Scotopic (dark-adapted) ERG waves are shown and represent the average of 10 flashes of high intensity light. The wave on the left is a normal scotopic ERG of 12 week old pig obtained one week prior to light exposure. The a-wave is the negative deflection with the trough indicated by the arrow. The amplitude of this wave is determined by measuring the depth of this trough from the baseline (arrowhead). The b-wave peak is indicated by the larger arrow. The amplitude of the b-wave is determined by measuring from the trough of the a-wave to the peak of the b-wave, in this case 108μV. On the right an ERG from the same eye of the same pig (16 weeks old) three weeks after light exposure is shown. Note the b-wave amplitude (168μV) has increased compared to the earlier value. This type of variation may be related to depth of anesthesia or due to maturation of the retina with a corresponding increase in b-wave amplitude. (x-axis 25μV/ division, y-axis 25 msec/ division)

Table 5: Biochemical data from control animals not exposed to light stress. Serum copper and zinc were analyzed from samples drawn pre and post light exposure. Calcium levels were determined on serum drawn at the end of the experiment post-light exposure. Copper and zinc levels were also determined in kidney and liver samples taken at sacrifice. ppm= parts per million

Animal	Diet	days on diet	pre Zn µg/g	post Zn µg/g	pre Cu µg/g	post Cu µg/g	L Zn µg/g	K Zn µg/g	L Cu µg/g	K Cu µg/g	Kidney	Liver	Ca mg/dl
											µg MT/ mg PR		
181	ZnN	13	0.73	0.93	1.77	1.48	46.98	37	15.93	5.39	0.25	0.76	8.45
7180	ZnN	13	0.58	0.71	1.31	1.51	56.43	29.8	3.9	7.14	0.26	1.70	7.95
110	ZnN	29	0.44	0.72	1.26	1.3	44.11	30.3	3.3	4.67	0.54	0.57	8.55
125	Zn-	12	0.46	0.31	1.28	1.77	44.88	28.4	3.55	2.87	0.18	0.76	7.65
111	Zn-	29	0.3	0.31	1.12	1.83	50.93	32.2	2.7	2.96	0.18	0.23	7.95
126	Zn+	11	0.48	0.5	1.39	1.92	44.77	35.2	3.55	4.41	0.26	0.54	8.45
109	Zn+	29	0.69	0.88	1.3	1.44	57.42	45.3	4.32	6.29	0.61	1.39	7.85

Table 6: Biochemical data from pigs in Part 1 of the study; 36 or 72 hours of intermittent light stress. Serum copper and zinc were analyzed from samples drawn pre and post light exposure. Calcium levels were determined on serum drawn at the end of the experiment post-light exposure. Copper and zinc levels were also determined in kidney and liver samples taken at sacrifice. When values could not be determined due to lost samples NA is in the cell.

Animal	Diet	days on diet	pre Zn μg/g	post Zn μg/g	pre Cu μg/g	post Cu μg/g	L Zn μg/g	K Zn μg/g	L Cu μg/g	K Cu μg/g	Kidney	Liver	Ca mg/dl
											μg MT/ mg PR		
115	Zn+	12	0.51	0.81	1.13	1.46	66.55	26.73	3.21	3.90	0.28	2.14	8.1
116	Zn+	14	0.56	1.14	2.08	1.48	61.6	25.3	3.93	2.87	0.42	1.55	8
124	Zn+	29	0.47	0.63	1.3	1.47	121.88	41.03	5.78	7.06	0.40	6.14	7.6
122	Zn+	32	NA	0.84	NA	1.2	68.2	33.22	5.26	5.69	0.33	1.51	7.5
121	Zn-	13	0.98	0.48	2.1	1.74	47.63	32.23	5.95	3.73	0.20	0.52	8
123	Zn-	15	0.53	0.63	1.64	1.61	54.01	32.34	4.41	3.55	0.27	1.26	7.9
119	Zn-	30	0.77	0.55	1.45	1.82	64.35	30.69	4.58	4.75	0.35	2.53	8
120	Zn-	32	NA	0.2	NA	1.39	28.05	26.29	3.04	2.44	0.17	0.16	7.8
117	ZnN	13	0.43	0.64	1.2	1.73	60.39	37.84	3.3	5.69	0.30	2.13	8.4
114	ZnN	16	0.45	0.63	1.2	1.51	36.74	37.84	3.55	6.03	0.22	0.47	8.5
112	ZnN	31	0.28	0.62	1.04	1.41	43.34	31.02	3.30	4.58	0.23	0.30	7.75
118	ZnN	31	NA	0.64	NA	1.25	46.31	28.71	2.7	8.00	0.45	1.12	7.6

Table 7: Biochemical data from pigs in Part 2 of the study; 72 hours of continuous light exposure. Serum copper and zinc were analyzed from samples drawn pre and post light exposure. Calcium levels were determined on serum drawn at the end of the experiment post light exposure. Copper and zinc levels were also determined in kidney and liver samples taken at sacrifice.

Animal	Diet	days on diet	pre Zn µg/g	post Zn µg/g	pre Cu µg/g	post Cu µg/g	L Zn µg/g	K Zn µg/g	L Cu µg/g	Kidney	Liver	K Cu µg/g	Ca mg/dl
										µg MT/ mg PR			
178	ZnN	13	0.64	0.95	1.33	1.22	65.79	35.08	43.78	0.34	1.36	12	9.05
363	ZnN	12	1.31	1.38	1.94	2.2	95.41	36.78	242.18	0.16	1.12	7.6	8.95
180	ZnN	13	0.87	0.8	1.25	1.46	68.71	33.63	11.07	0.32	2.74	4.9	8.75
361	ZnN	31	1.05	1.13	1.75	1.33	83.77	37.03	14.85	0.53	4.53	11	9.35
179	Zn-	13	0.77	0.91	1.64	1.29	64.33	30.71	17.01	0.02	0.26	14	9.05
182	Zn-	13	0.89	0.89	2.11	1.44	35.56	26.09	9.17	0.18	0.36	3.2	9.65
364	Zn-	13	0.95	1.16	1.85	1.96	80.85	38.48	10.8	0.06	1.05	6.2	8.95
362	Zn-	31	0.95	0.96	1.55	1.44	47.47	32.89	161.07	0.13	1.10	4.3	8.75
365	Zn+	13	0.71	1.05	1.83	1.42	66.28	30.95	39.73	0.23	1.07	3.8	8.95
176	Zn+	13	0.55	0.8	2.07	1.05	62.39	31.2	12.96	0.36	1.64	6.2	9.35
177	Zn+	13	0.77	0.73	1.81	1.38	65.31	32.16	14.85	0.19	1.81	5.1	8.55
366	Zn+	31	0.93	1.09	1.68	1.77	126.72	39.45	18.37	0.78	6.27	9.4	9.75

Table 8: Summary of liver and kidney Zn and MT levels for control animals and those exposed to intermittent or continuous light. Feeding the diets did not significantly influence the status of the animals after short (S) term feeding for 11-13 days. When diets were fed for a longer period (L) of 29-31 days, a trend towards increasing tissue Zn and MT content with increased dietary zinc level is often present. In the control group two animals were fed the ZnN diet so the values were averaged. The values for the 36 and 72 hour intermittent exposure groups were averaged so in the short term row in the intermittent group n=2. In the continuous short term groups 3 animals were in each diet group. Otherwise all values represent those from one animal.

		Kidney						Liver					
		Zn (µg/g)			MTµg / mg Protein			Zn (µg/g)			MTµg / mg Protein		
		Zn-	ZnN	Zn+	Zn-	ZnN	Zn+	Zn-	ZnN	Zn+	Zn-	ZnN	Zn+
Control	S	28.4	33.4	35.2	0.18	0.26	0.26	44.88	51.71	44.77	0.76	1.23	0.54
	L	32.2	44.1	45.3	0.18	0.54	0.61	50.93	44.1	57.42	0.23	0.57	1.39
Int.	S	32.29	37.84	26.02	0.24	0.26	0.37	50.82	48.57	64.08	0.89	1.3	1.85
	L	28.49	29.87	37.13	0.26	0.34	0.4	46.2	44.82	95.04	1.35	0.71	3.83
Cont.	S	31.76	35.16	31.43	0.09	0.35	0.26	60.25	76.64	64.66	0.56	1.74	1.51
	L	32.89	37.03	39.45	0.13	0.53	0.78	47.47	83.77	126.7	1.1	4.53	6.27

different diets for 11-13 days or for the animals fed the diets for 3 weeks. The small number of control animals made it difficult to compare the control vs. the exposed groups with parametric tests such as the analysis of variance (ANOVA), so the non-parametric Kruskal-Wallis (KW) test was also performed on the data.

At time of sacrifice, the mean serum zinc concentrations for the control animals fed the Zn-, ZnN or Zn+ diet for 11-13 days were respectively 0.31, 0.82 and 0.52 ppm. Statistical analysis by the ANOVA and KW tests revealed no significant difference between the three group means, p = 0.34 and p = 0.26. In the continuously exposed group the serum zinc levels for the Zn-, ZnN and Zn+ fed animals were 0.98, 1.04, and 0.86. Again no significant difference between the three groups was present (ANOVA p = 0.60 and KW p = 0.51).

Copper and zinc levels in the liver and kidney were determined by atomic absorption spectrophotometry and are also shown in Tables 5-7. There was no significant difference in the level of these elements between the different diet groups. The liver zinc levels for control animals fed the Zn-, ZnN or Zn+ diets for 11-13 days were respectively, 44.88 (n=1), 51.71 (n=2) and 44.77 (n=1) ppm. The ANOVA and KW tests revealed no significant difference (p = 0.70 and p = 0.26). The kidney zinc levels in control animals fed the low, normal and high levels of dietary zinc for 11-13 days, 28.4 (n=1), 33.4 (n=2) and 35.2 (n=1) ppm were not significantly different (ANOVA p = 0.71 and KW p = 0.41).

Kidney levels of Zn and MT are also shown in Table 5-7. Kidney MT levels, unlike liver MT, are a good indicator of zinc status that is not as affected by variables such as stress or disease.[122] For control animals fed the Zn-, ZnN and Zn+ diets, the kidney MT levels were 0.18, 0.26 and 0.26 μg MT/ mg protein. The ANOVA and KW tests revealed no significant difference between the groups ($p = 0.11$ and $p = 0.35$). In the continuously exposed group the kidney MT levels in the Zn-, ZnN and Zn+ fed animals were 0.09, 0.35 and 0.26 μg MT / mg protein. The ANOVA and KW showed that the three means were not significantly different $p = 0.08$ and $p = 0.09$. The differences however approached significance suggesting that in the continuously light exposed group, the animals fed the zinc deficient diet had lower kidney MT levels when compared to those fed the normal and high zinc diets. It is not surprising that the animals fed the high zinc diet did not have any significant alteration in status because the experimental diet did not have the desired level of 300 ppm zinc but had only 240 ppm zinc.

Feeding the different levels of dietary zinc for three weeks appeared to influence the liver and kidney zinc and MT levels to a greater extent than feeding for 11-13 days. In several cases if liver and kidney Zn and MT values of the animals fed diets for 11-13 days vs. 29-31 days are compared it is appears that there is some influence of diet, i.e., values from animals fed the Zn- diets are low, ZnN intermediate and Zn+ higher (Table 8). In animals fed the diets for 29-31 days statistical analysis could not be performed because only one animal was in each diet or light group.

If the data in Tables 5-7 is closely examined certain animals may be identified that had significant changes in the Zn and Cu in serum, liver, or kidney, or that had changes in metallothionein levels that suggest some alteration in zinc status. In the control group it appeared that #125 fed the Zn- diet for 11 days had a decrease in serum Zn, however, when the liver and kidney Zn levels are examined no significant decrease in Zn content was apparent. The kidney MT level however was relatively low compared to the rest of the population at 0.18 µg MT / mg protein. Similar tissue levels of serum and organ Zn and MT are present in #111, the animal fed the Zn- diet for 29 days. The values suggest both of these animals had a mild negative Zn balance. Animal #109, fed the Zn+ diet for 29 days, had positive changes in serum Zn, liver and kidney Zn and in MT (0.61 µg MT/mg PR) suggesting that feeding the Zn+ diet (with 300 ppm Zn) for 29 days induced a supplemented state.

Examination of the intermittent light diet groups revealed a similar trend in increasing kidney MT levels with increasing amount of dietary zinc. The mean kidney MT levels for the animals fed the low, normal and high zinc diets (n=2 per diet group) were 0.24, 0.40, and 0.35 µg MT / mg protein. Certain animals were identified that appeared to have altered status. Animals #120 and 121 had low kidney MT values. In animal #120, which also had decreased serum, liver and kidney Zn compared to the rest of the population, the low dietary zinc level probably induced a mild deficiency after 32 days of feeding. The zinc deficiency however appeared marginal as no overt clinical symptoms such as parakeratosis were observed.

In the continuously exposed group the trend of increasing kidney MT with increasing dietary zinc level again was apparent. Very notable was the low kidney MT level in animals #179 and 364 both which were fed the Zn- diet for 13 days. Serum, liver, and kidney zinc levels, however, did not substantiate significant low zinc status. Animal #182 had low liver and kidney zinc and somewhat low kidney MT compared to the control groups' values which again may indicate a mild negative zinc balance. In contrast animal #366 seemed to have a positive alteration in Zn status after being fed the Zn+ diet for 31 days since serum, liver, kidney zinc and MT levels were greater than that of the rest of the population.

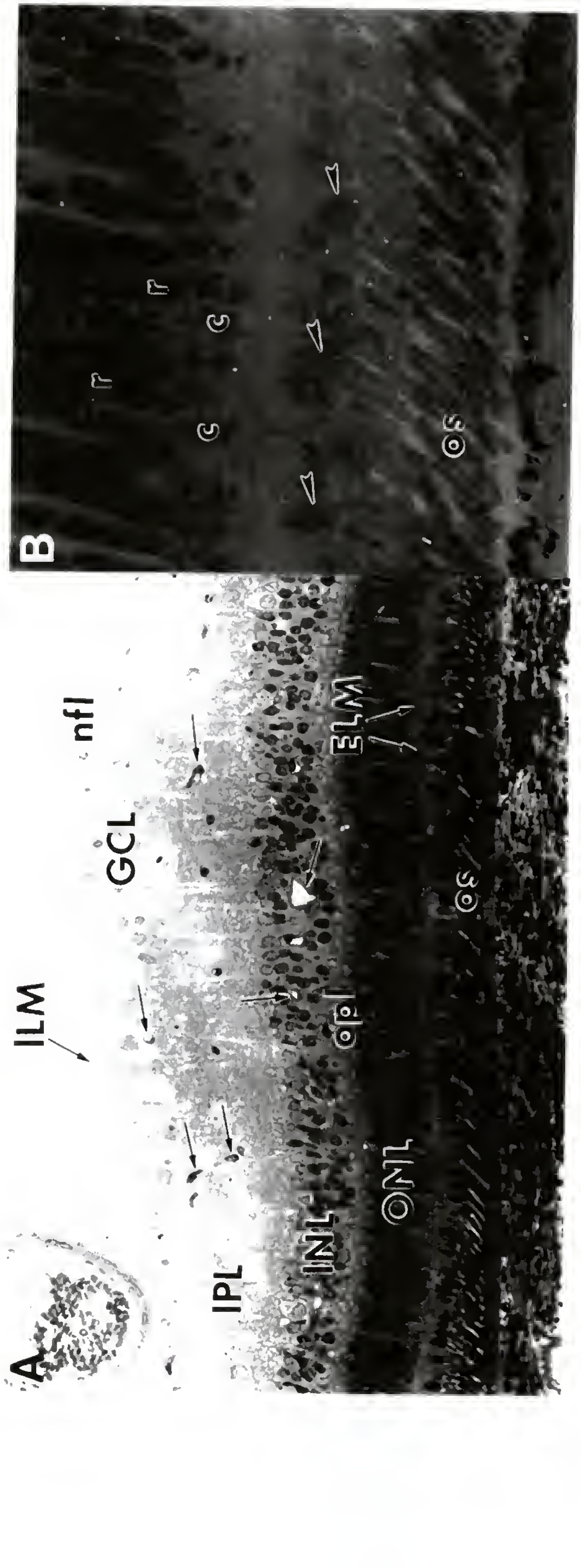
Normal Porcine Retina (unexposed)and Control Animals

The normal central porcine retina in the inferotemporal quadrant had a mean retinal thickness of 272.7 µm. The outer nuclear layer (ONL) averaged 39.8 µm in thickness, which was an average of 6-7 nuclei in height. The ONL was usually a little thicker than the inner nuclear layer (INL). Cones were numerous and appeared as a layer of euchromatic nuclei just internal to the external limiting membrane (Figure 6). The staining pattern of rods and cones differed; the rod nuclei were more heterochromatic than cone nuclei (Figure 7). Internal to the ONL the cone pedicles were very prominent. The ellipsoid regions (containing mitochondria) of porcine cone inner segments (IS) were large, plump, and stain densely with toluidine blue (Figure 6). Ultrastructurally rod and cone IS were packed with mitochondria and the rod IS was longer and narrower than that of the cone.(Figure 7). The mitochondria in the IS of both types of photoreceptors had numerous cristae indicative of a high metabolic rate.

Sections were made from the peripapillary to the peripheral retina in the inferotemporal wedge of eye tissue from a control animal on a normal Zn diet. The nerve fiber layer progressively thins from the optic nerve towards the periphery. Larger arterioles and veins were found in this layer (Figure 6). Small vessels and capillaries were found in the ganglion cell layer (GCL), the middle of the inner plexiform layer (IPL) and within the INL. The ganglion cell layer contained small, medium and large cells. The density of ganglion cells decreased towards the periphery. The INL nuclear thickness also decreased

Figure 6: Normal porcine retina.

a) The smaller vessels (arrows) are in the ganglion cell layer (GCL), the middle of the inner plexiform layer (IPL) and in the outer border of the inner nuclear layer (INL). (800x) b) Note the difference in staining of rod (r) and cone(c) nuclei in the ONL. Cone nuclei are oval, adjacent to the external limiting membrane (ELM), and are more euchromatic. The cone ellipsoids are prominent (arrowheads). The OS disk lamellae are well organized and in close apposition with the RPE microvilli. (2000x) OS- outer segments. ONL-outer nuclear layer, OPL - outer plexiform layers, INL-inner nuclear layer, NFL-nerve fiber layer, ILM-internal limiting membrane.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ONL
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opl
GCL
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ELM
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B
c
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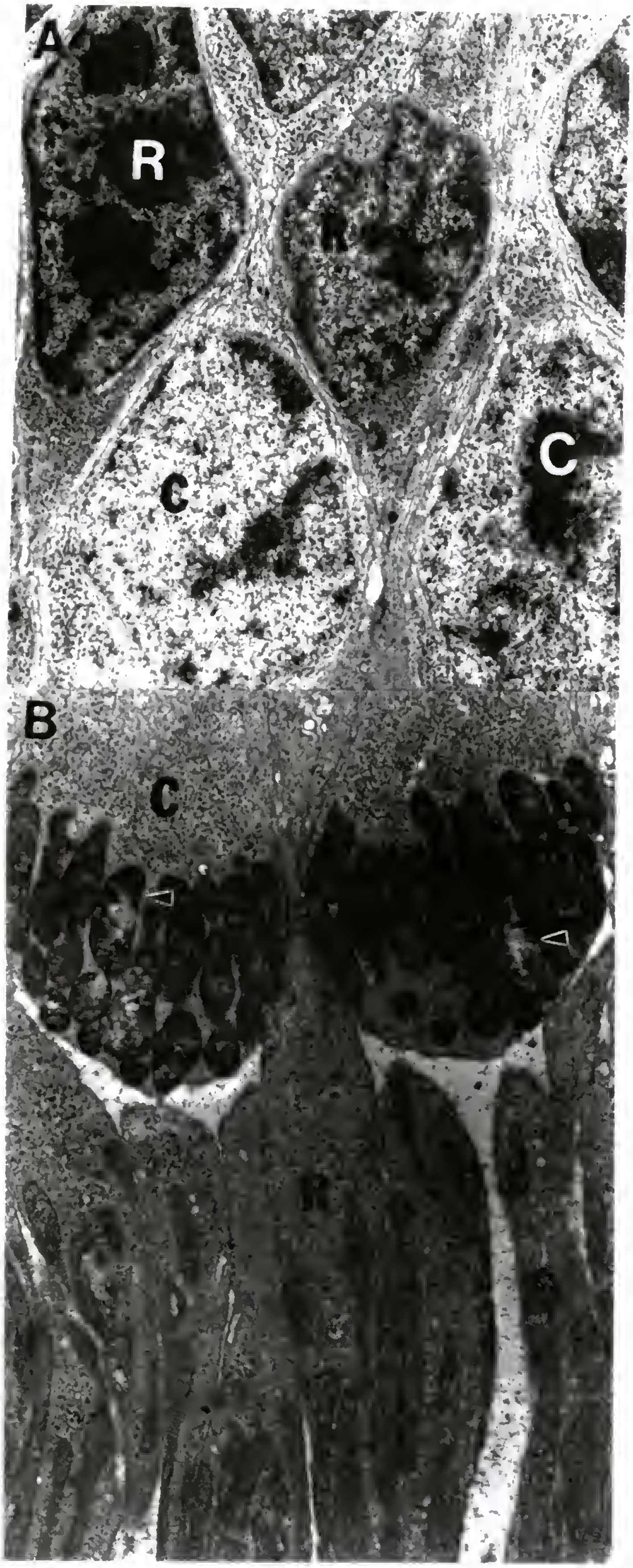
c
r
os

Figure 7: Outer retinal layers of normal porcine retina.

a) Electron micrograph of the ONL layer of a control animal fed the ZnN diet. The rod nuclei (r) have more heterochromatin than the cones (c). Note lack of intercellular space. (8000x) b) Rod (r) and cone (c) inner segment ellipsoid regions are packed with mitochondria. The cone IS are short and plump compared to the long narrow rod IS. Note that the mitochondrial cristae in most instances are easily differentiated and very numerous, indicative of the high metabolism of the photoreceptors. Some mitochondria are disrupted (arrowheads) but the infrequency of this observation indicates that it is an artifact. (8000x)


A
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from the optic nerve to the periphery. ONL thickness decreased as the distance from the optic nerve increased. The ONL, in number of nuclear layers, varied as follows: peripapillary, 6-7; central near optic nerve (paracentral), 5-6; central, 4-5; and peripheral, 4-5. The ONL was notably thicker than the INL in the peripapillary retina. In the central section near the optic nerve, the INL thickness approximated that of the ONL. In the central section nearer the periphery and the peripheral retina, the ONL was thicker than the INL. The number of cones decreased from central to periphery. ON this inferotemporal wedge, the rod to cone ratio in the ONL varied as follows: peripapillary, 8-9:1; paracentral,6-7:1; central 5-6:1; and peripheral, 8:1.

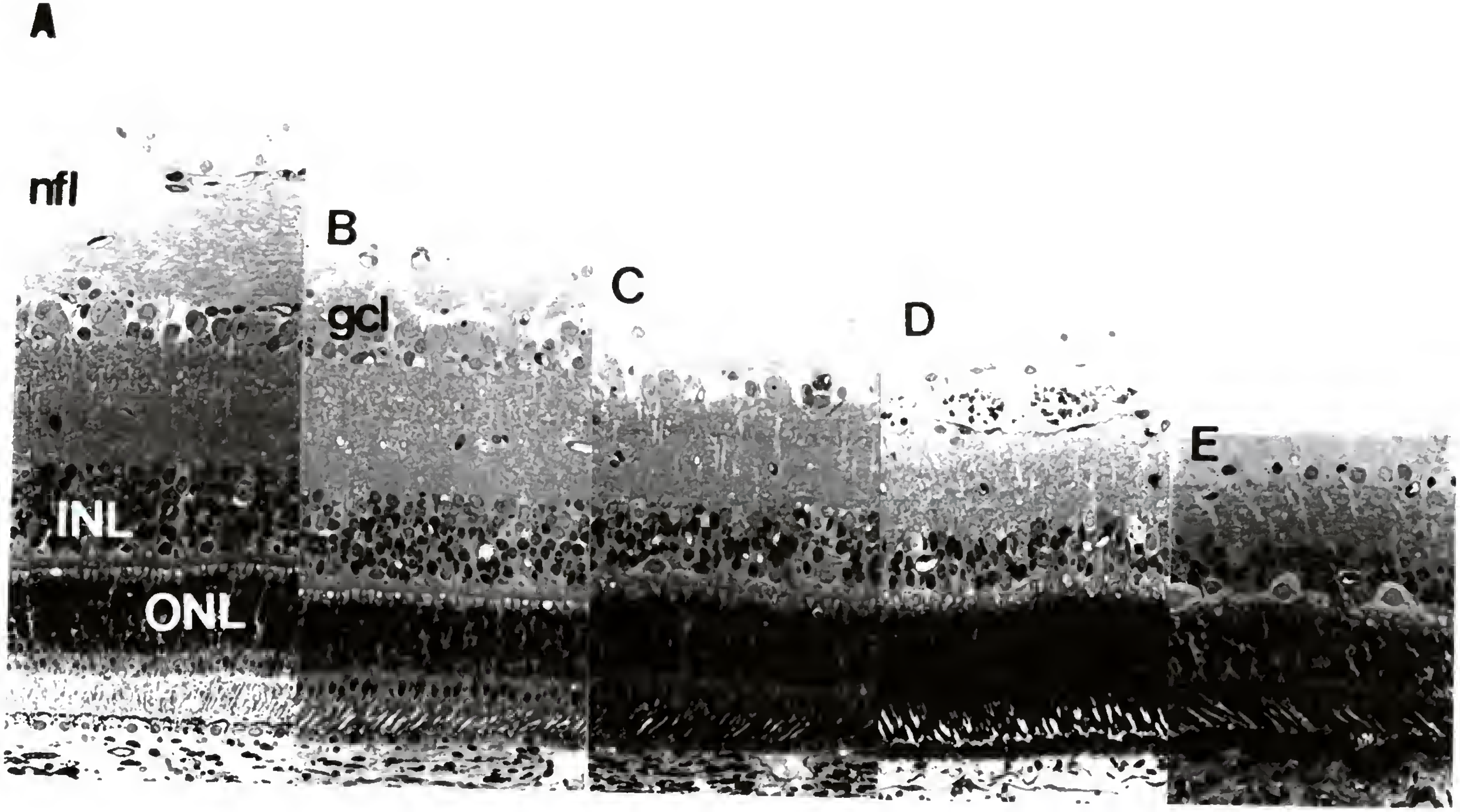
A similar series of central to peripheral sections were taken from a control animal's superotemporal retina (Figure 8). The central area of this wedge represented the area centralis. The NFL and vessels were similarly distributed as the inferotemporal wedge. The GCL in peripapillary and central regions in this wedge was thicker than the inferotemporal wedge. The number of ganglion cells again decreased in the peripheral sections.

The area centralis (AC) can be identified by several histologic features. As the area centralis is approached, the inner nuclear layer becomes equal to or greater in thickness than the ONL. The GCL is 2-3 layers of densely packed cells. The rod to cone ratio in the AC decreases to 4.4:1 indicating a relative increase in cone density. The average rod to cone ratio is 6.7:1 in the inferotemporal quantrant and 5.5:1 in the superotemporal quadrant substantiating the presence of a cone rich area in the superotemporal quadrant.

In the superotemporal wedge, similar to the inferotemporal wedge, the ONL thickness progressively decreased towards the periphery; being 6-7 nuclei thick near the

Figure 8: Histology of the normal porcine retina. A series of sections from the optic nerve to the periphery are shown. All photographs were taken at the same magnification, 200x and after enlargement are magnified 400x. From a-e note the progressive thinning of the entire retinal thickness and it nuclear layers.

a) Normal peripapillary retina. The NFL is thick with numerous ganglion cells in the GCL. The ONL and INL are thickest in this region and approximately equal in thickness. b) Central section adjacent to optic nerve section shown in (a). The NFL layer is thinner but ganglion cells, INL and ONL cells are still abundant in this central area. Note the INL is slightly thicker than the ONL and the GCL is two to three cells thick. This section is representative of the porcine area centralis. Compare the numerous ganglion cells here to the less abundant GCL in the central inferotemporal section in Figure 6. c) Central section adjacent to (b) is in the mid-peripheral retina. The GCL layer is not as prominent, the INL and ONL are thinner than more central sections and cones (can estimate by counting ellipsoids) are somewhat less concentrated. d) The adjacent peripheral retina has thinner ONL and INL. Ganglion cells are much less numerous. Cones are still fairly well concentrated in this mid-peripheral section from the superiotemporal retina. The concentration of cones is similar to that seen in the central section of the inferiotemporal wedge shown in Figure 6. e) This section was taken from the most peripheral edge of the wedge. Some artifactual changes in RPE are present due to the razor blade cuts made to harvest the wedge. Note the thinner ONL and INL. Few ganglion cells are observed and the NFL is very thin.


A
nfl
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gcl
C
D
E
INL
ONL

optic nerve, 5 in the central area near the optic nerve, 4 in the central area nearer the periphery and 4 in the periphery. The rod to cone ratio in the area centralis usually was between 4-5:1. From the optic nerve to the periphery the ratio changes from 8-9:1 near the optic nerve, 5.5:1 in the paracentral sections, 4.4:1 in the central section (with AC), and 5.4:1 in the periphery.

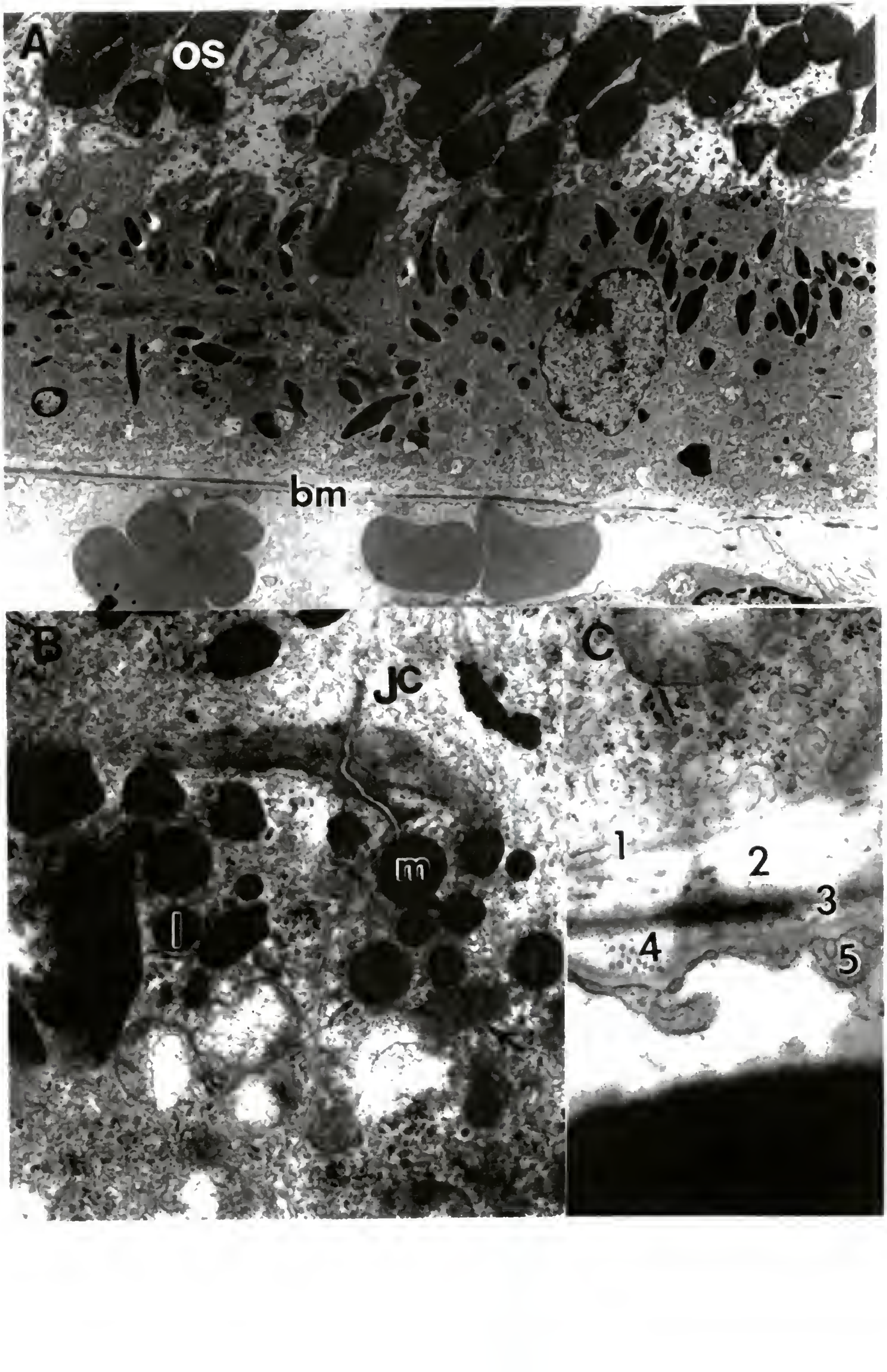
The average retinal pigment epithelial height in the inferotemporal quadrant was 8.9 µm. Ultrastructurally the RPE cytosol contained organelles typically found in the RPE of other species including: melanin granules, residual bodies, phagocytized OS material, lysosomes, golgi apparatus, and smooth and rough endoplasmic reticulum (Figure 9). The basal cell membrane has some infolding but this was not a prominent feature as seen in other species. The nucleus is large, oval, and euchromatic. The pig has a well developed Bruch's membrane with five layers in areas adjacent to the choriocapillaris: basement membranes of RPE, inner collagenous zone, elastic fiber layer, outer collagenous zone and the basement membrane of endothelium of the choriocapillaris.

Control Lighting (14 days on diet)

Normal zinc diet. This animal received the corn-soybean based diet for 14 days. Retinas of both eyes of this animal were normal. Rarely, a cell was found degenerating and moving through the external limiting membrane (ELM). Some ONL nuclei had an altered chromatin appearance with a rounding up of the nucleus and more euchromatin. The left eye of this animal had a moderate number of immature melanosomes in the RPE indicative of melanogenesis.

Figure 9: Normal porcine RPE-OS interface.

a) The OS are well embedded in the microvilli of the apical RPE. The cytosol of a normal cell is dense due to the large amount of smooth endoplasmic reticulum that fills all extra space. (8000x) b) Higher magnification of RPE cytosol demonstrates the typical apical junctional complex (jc) that represents the outer blood retinal barrier. Other organelles are evident: melanin granules (m), lysosome (l), and mitochondria (arrow). (20,000x) c) Normal Bruch's membrane adjacent to a capillary of the choriocapillaris. The membrane has five well developed layers as in humans: 1) basement membrane of the RPE, 2) inner collagenous zone, 3) elastic fiber layer, 4) outer collagenous zone, and 5) basement membrane of capillary endothelium. (30,000x, TEM)


A
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5

Zinc deficient diet. This animal received a zinc deficient casein-sucrose based diet for 14 days. Mild disorganization of the outer segments was apparent at the light microscopic level and confirmed by transmission electron microscopy. Displacement of IS into outer segments was seen. On ultrastructural examination, IS were swollen and abnormally shaped with some degenerative mitochondria (Figure 10a). Most nuclei in the ONL were normal except for an occasional degenerating nucleus observed in the ELM. Melanogenesis was observed in the left eye of this animal and incompletely melanized granules were very numerous in several RPE cells. The other eye of this animal did not exhibit melanogenesis.

Zinc supplemented diet. This animal received a zinc supplemented corn-soybean diet for 14 days. The retina was normal except for an occasional degenerating cell located in the ELM. One IS was observed between the RPE-OS interface and mild OS disorganization was observed in some areas. Evidence of melanogenesis was found in the left eye of this animal. In this eye immature melanin granules were infrequently observed.

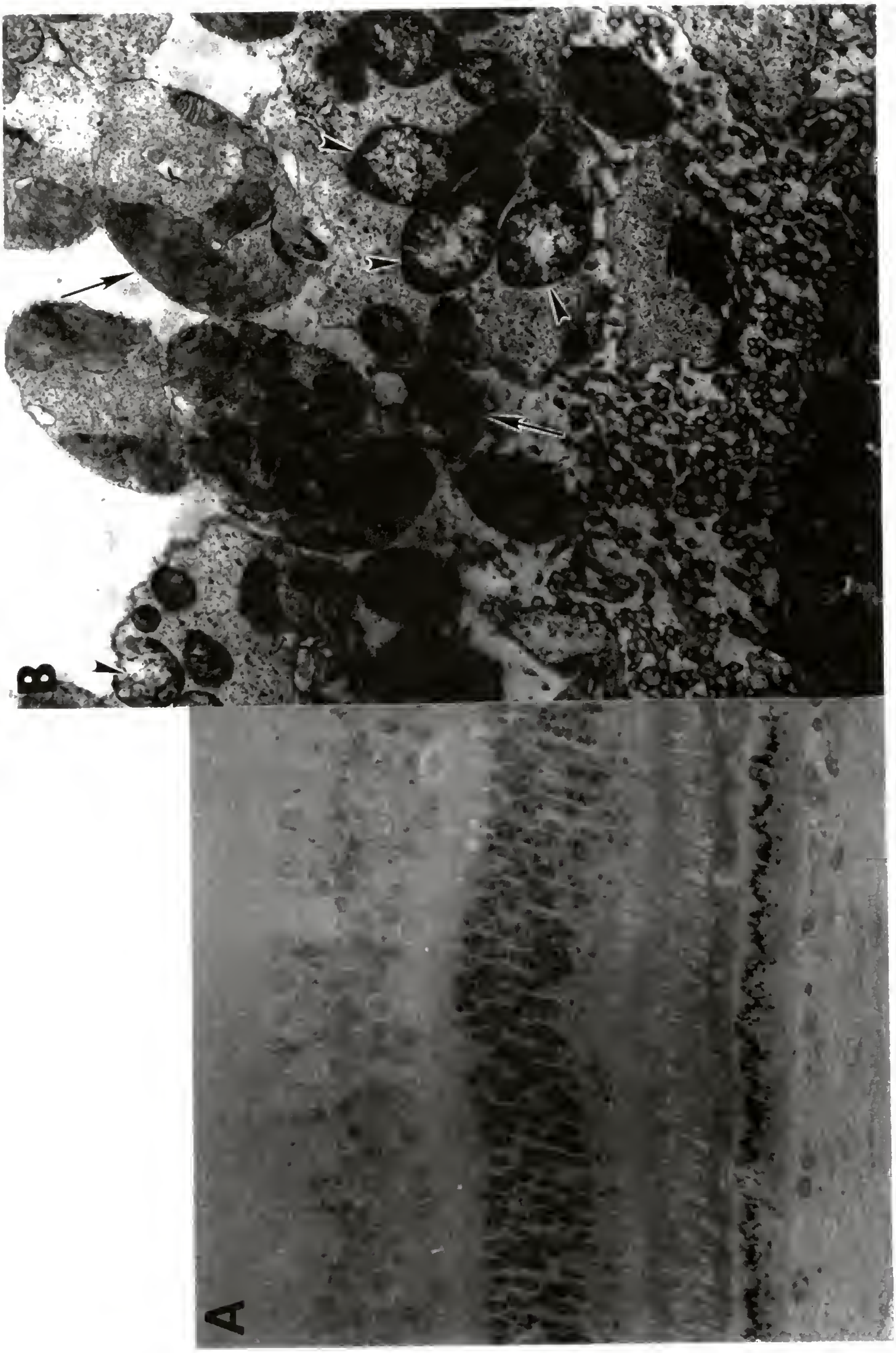
Control Lighting, (33 days on diet)

Normal zinc diet. This animal received a corn-soybean based diet for 33 days. The retinas of this animal were normal. Immature melanosomes with visible melanofilaments indicative of melanogenesis were observed in both eyes of this animal. In one eye the immature granules were infrequently observed.

Zinc deficient diet. This animal received a zinc deficient casein-sucrose based diet for 33 days. The retinas were normal with minimal evidence of degeneration. There was the occasional focus with disrupted OS and a few pyknotic nuclei in the ONL (Figure 10b). The

Figure 10: Control (no light exposure) animal fed the Zn- diet for eleven days.

a) Outer retina of a control pig fed the zinc deficient diet for 33 days. There appears to be poor attachment of the OS to the RPE (compare to Figure 6). The inner retinal layers are normal.(800x) b) Some disruption of OS was present (arrows) and displacement of IS into the OS layer has occurred. Inner esgments with swollen mitochondrial (arrowheads) are present on the surface of the RPE microvilli. (10,000x)


B
A

inner retina was normal. The outer segment- RPE interface appeared loose as the OS were not well embedded in the RPE microvilli. Some disorganization of OS and some degenerative IS were be observed. No melanogenesis was observed in either eye of this animal.

Zinc supplemented diet. This animal received a zinc supplemented corn-soybean based diet for 33 days. The retinas of this animal were normal. Immature melanosomes were observed infrequently in one eye of this animal suggesting some melanogenesis was ongoing.

Photic Injury

Animals were observed twice daily while in the photic injury unit and did not exhibit signs of stress. Behavior observed included laying down and sleeping, or walking around the cage playing with the food dishes or water supply. Because the unit was elevated off the floor to allow for cleaning, the pigs were able to and were often observed laying near the edge of the unit and partially shading one eye by positioning their eyes under or near the edge of the bottom of the unit.

Pilot Study: 36 and 72 hours of Intermittent Light and Heat Stress

The intermittent lighting schedule was 18 hours light and 6 hours dark with light onset at 8 a.m. As mentioned previously, during the pilot study the temperature in the unit averaged 90-93°F and animals exhibited a tendency towards hyperthermia with rectal temperatures of 105-106°F compared to the normal range of 101.6-103.6°F.[176] Several studies have demonstrated that elevated body temperature (hyperthermia) accentuates photic injury [3,43,49]. To minimize the number of experimental variables that might influence photic injury, in further trials, a plastic shield was used to absorb and reflect heat, and along with fans maintained the temperature in the unit at 80°F.

The retinas of animals exposed to 36 and 72 hours intermittent light stress and sacrificed 72 hours after exposure were severely damaged. The type of damage was similar in both animals but subjectively it was more severe in the animal exposed to 72 hours of intermittent light.

Damage was diffuse and present in all retinal layers (Figure 11). In the animal exposed to 72 h of intermittent light, some ONL nuclei were pyknotic but most appeared swollen. All nuclei in the ONL exhibited chromatolysis with loss of distinct chromatin (Figure 12). In both animals, damage to the inner retina was less pronounced and limited to a generalized loss of staining which was interpreted as an edematous response. Some INL cells were pyknotic; again this occurred more frequently in the animal exposed to 72 hours of intermittent light (Figures 11 and 12).

Inner segment mitochondria were disrupted and swollen. The outer segments appeared fairly normal with only some exhibiting disorganization. In the animal exposed to 36 hours of intermittent light the RPE appeared normal other than a few swollen cells. In the animal exposed to 72 hours of intermittent light, RPE cells were tall and had vacuolated cytoplasm suggestive of intracellular edema (Figure 12). Many mitochondria in the RPE cytosol appeared swollen.

Part 1: Intermittent Light Stress (36 hours): 72 hours Post-Exposure (12 days on diet)

Three of six eyes examined had significant injury. The other 3 eyes had no damage or only mild and often focal degeneration that was not significantly different from controls.

Figure 11: Severely damaged retina of light-exposed (72 hours intermittent) and heat-stressed animal.

a) Diffuse retinal edema is present. Several nuclei in the INL are pyknotic (arrowheads). OS are disrupted, the RPE layer is tall due to edema and the RPE-OS interface is compromised. (800x) b) Most nuclei in the ONL are swollen or pyknotic and the ellipsoid region of the IS are edematous (arrows). Nearly all the IS of the rods exhibit swollen mitochondria with this change occurring less frequently in the cones. In many RPE cells, the cytosol appears vacuolated (arrowheads). (800x)

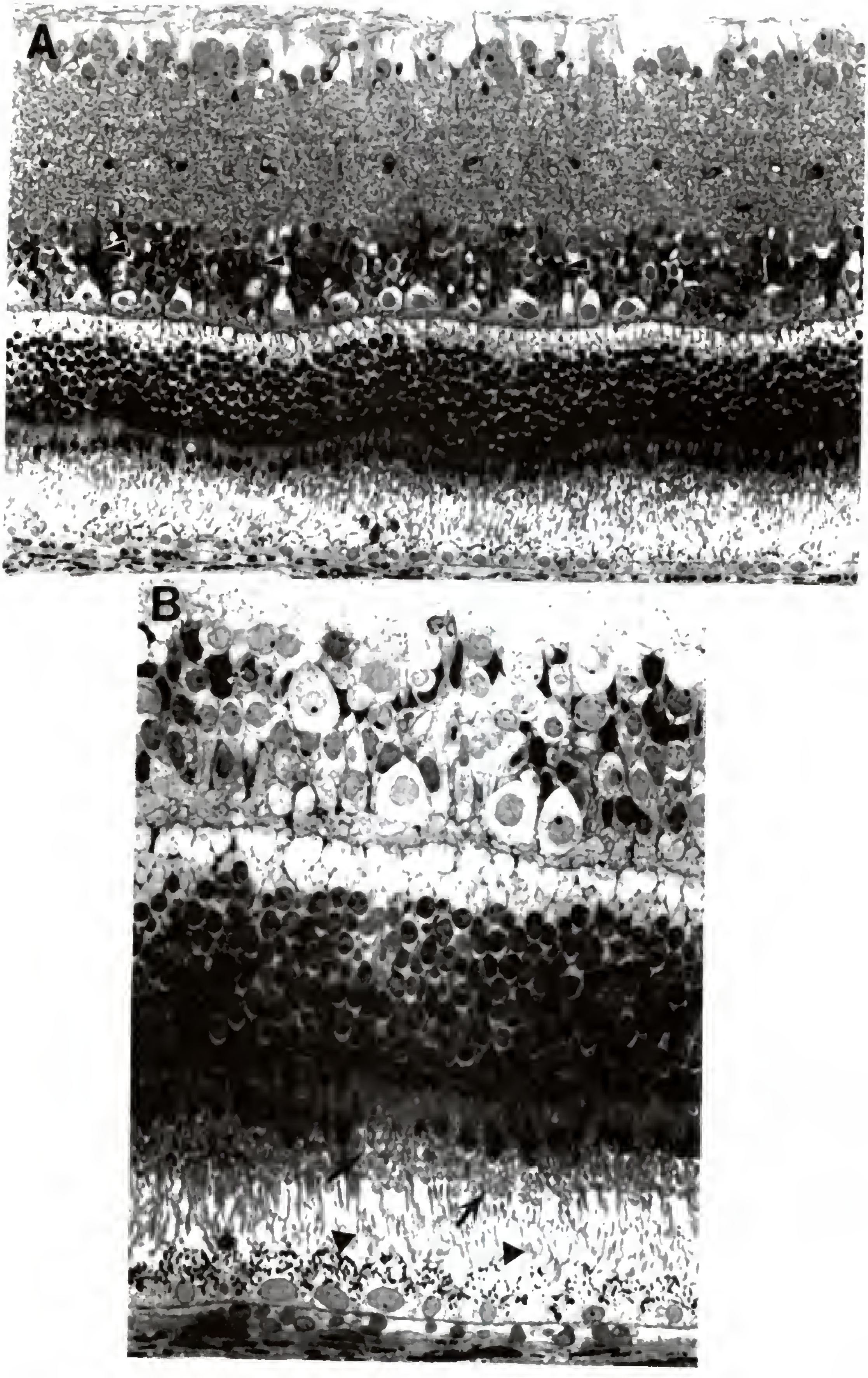

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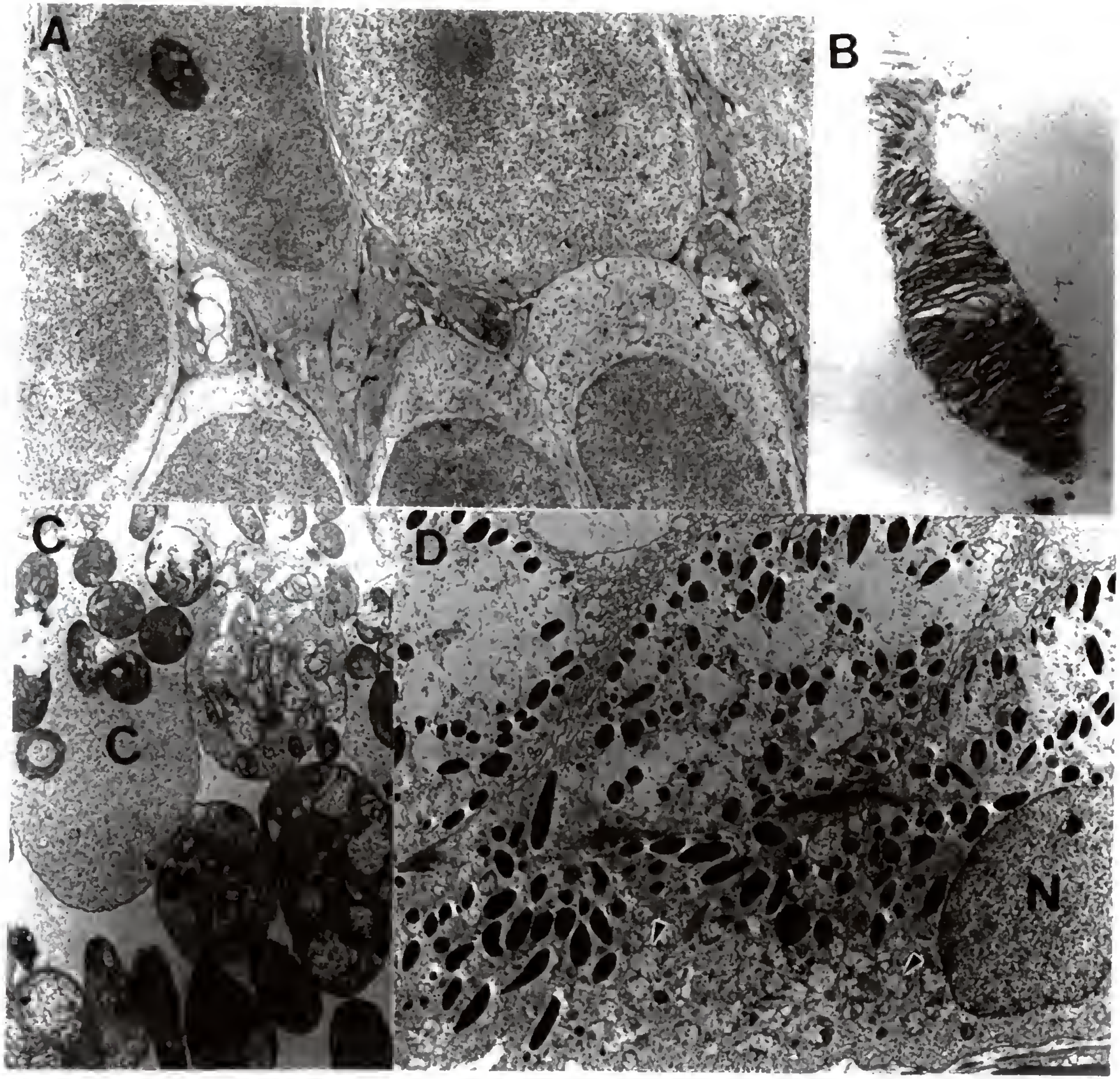
Figure 12: Outer retinal changes after 72 hours of intermittent light and high body temperature.

a)Diffuse chromatolysis of ONL nuclei is apparent. (10,000x) b) Remarkable was the insensitivity of the OS to disruption compared to the IS. Disorganization of OS was observed infrequently. (20,000x) c) Inner segment mitochondria (m) are swollen and disintegration of cristae was observed. The mitochondria in the cone (c) IS appeared more viable as the cristae pattern is nearly normal although both rods (r) and cones are degenerative. (10,000x) d) The RPE was also adversely affected by light and heat. Note loss of the dense cytoplasmic staining due to edema. The nucleus (n) has less heterochromatin than usual. Several mitochondria are swollen (arrowheads). Melanin granules appear normal. (6000x)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Two of the three damaged eyes were from the animal fed the zinc normal diet. One eye of the animal fed the zinc supplemented diet showed significant damage.

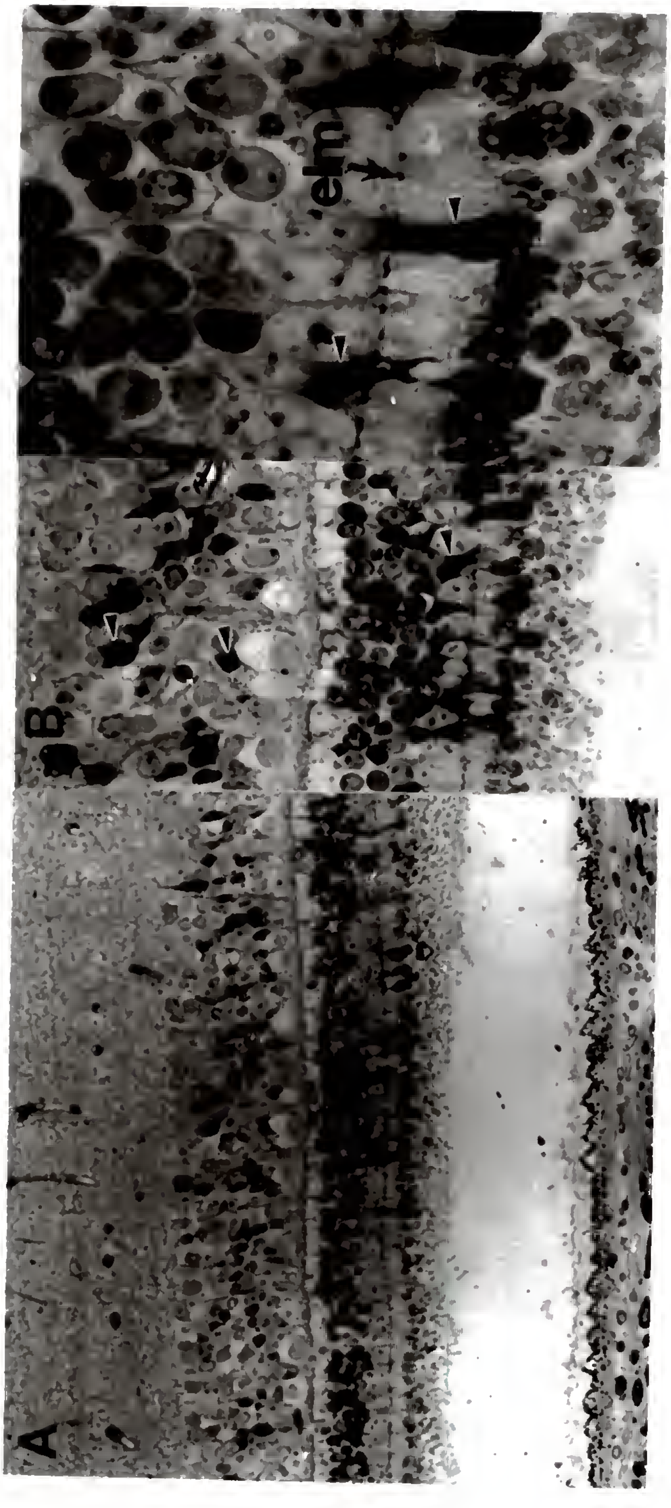
Normal zinc diet. Moderately severe damage occurred in both eyes of this animal. The retina was diffusely edematous and artifactually detached presumably due to poor attachment between the RPE and OS. If the attachment between the RPE and OS is firm, artifactual detachment usually results in torn OS and remnants of OS are left on the surface of the RPE. Therefore, when these changes are not observed it usually indicates poor RPPE-OS attachment. Multiple nuclei in the ONL were pyknotic and located in the ELM (Figure 13). Some INL nuclei were pyknotic. In some areas, inner and outer segment degeneration was present. Rarely, the occasional RPE cell was degenerating with vacuolation of the cytosol or pyknosis of the nucleus.

Zinc deficient diet. The retina was normal except for one focus of moderate degeneration at one end of the section. In this degenerative area, changes observed were similar to that found in the animal on the zinc normal diet. Multiple nuclei in the ONL were pyknotic and located in the ELM. Some OS lamellae were disorganized and the mitochondria in IS were unrecognizable due to swelling and disruption of normal architecture. Three other regions of this eye were examined and appeared normal so this focus of degeneration was unlikely to have been induced by light. The other eye of this animal was normal.

Zinc supplemented diet. The left eye of this animal exhibited very mild damage manifested as alteration of the chromatin pattern of several nuclei in the ONL (Figure 14a). Affected rod nuclei were more euchromatic and rounded compared to normal nuclei in

Figure13: Moderately severe retinal damage in an animal fed the ZnN diet and exposed to 36 hours of intermittent light without heat stress and sacrificed three days post-exposure.

a) Note diffuse retinal edema, occasional INL pyknosis and more frequent ONL pyknosis. (400x) b) Many nuclei in the INL and ONL exhibit pyknosis (arrowheads). If not pyknotic, ONL nuclei exhibit swelling and chromatolysis. (800x) c) Most rod and cone OS and IS are swollen. Movement of cells outward through the ELM is frequently observed when ONL cells die. (2000x)


A
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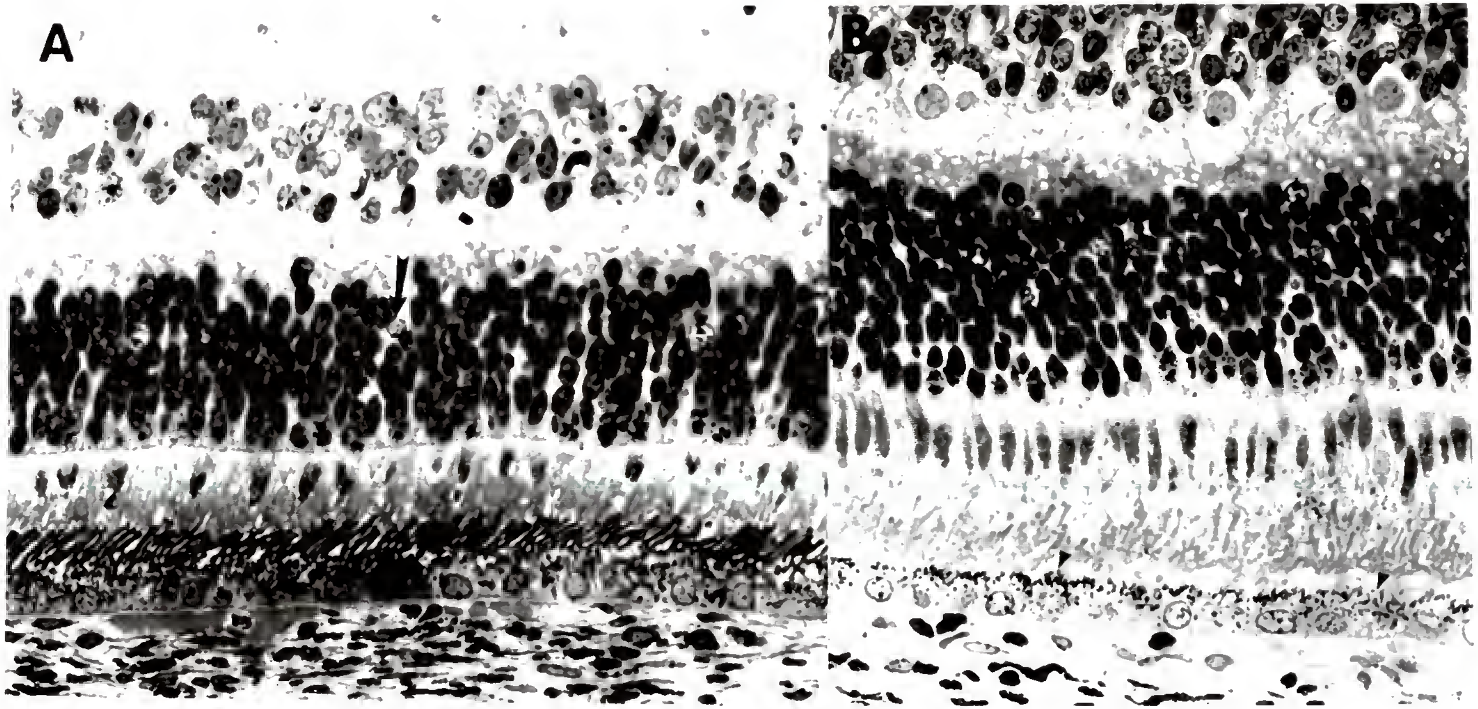
elm

Figure 14: Asymmetric retinal damage in an animal exposed to 36 hours of intermittent light, fed the zinc supplemented diet and sacrificed 3 days post-exposure.

a) The left eye of this animal was normal except for frequent ONL nuclear chromatin alterations (arrows) This is a more peripheral section with less numerous cones (fewer cone ellipsoids). (800x) b) The right eye of this animal exhibited moderate damage with swelling of the INL, multifocal ONL pyknosis, ONL chromatin alterations and disruption of rod IS. The RPE-OS interface appeared loose and many RPE cells were vacuolated (arrowheads). (800x)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control eyes. No nuclear displacement was noted and the rest of the retinal layers were normal. The right eye exhibited mild damage (Figure 14b). Numerous cells were undergoing pyknosis and moving through the ELM. Several RPE cells appeared tall and vacuolated.

Part 1: Intermittent Light Stress (36 hours): 3 weeks Post-Exposure (30 days on diet)

Zinc normal diet. Both eyes of this animal had very mild signs of degeneration not significantly different from that observed in some control animals' retinas. The occasional rod nucleus was euchromatic and rounded. Some vacuolated IS were observed in the ONL (Figure 15a). Subjectively, in some areas there appeared to be a decrease in ONL density. These findings suggest that prior light-induced damage had been partially repaired.

Zinc deficient diet. Both retinas appeared normal. In one eye normal appearing IS were observed on the surface of the RPE suggesting prior displacement of unhealthy IS which then recovered.

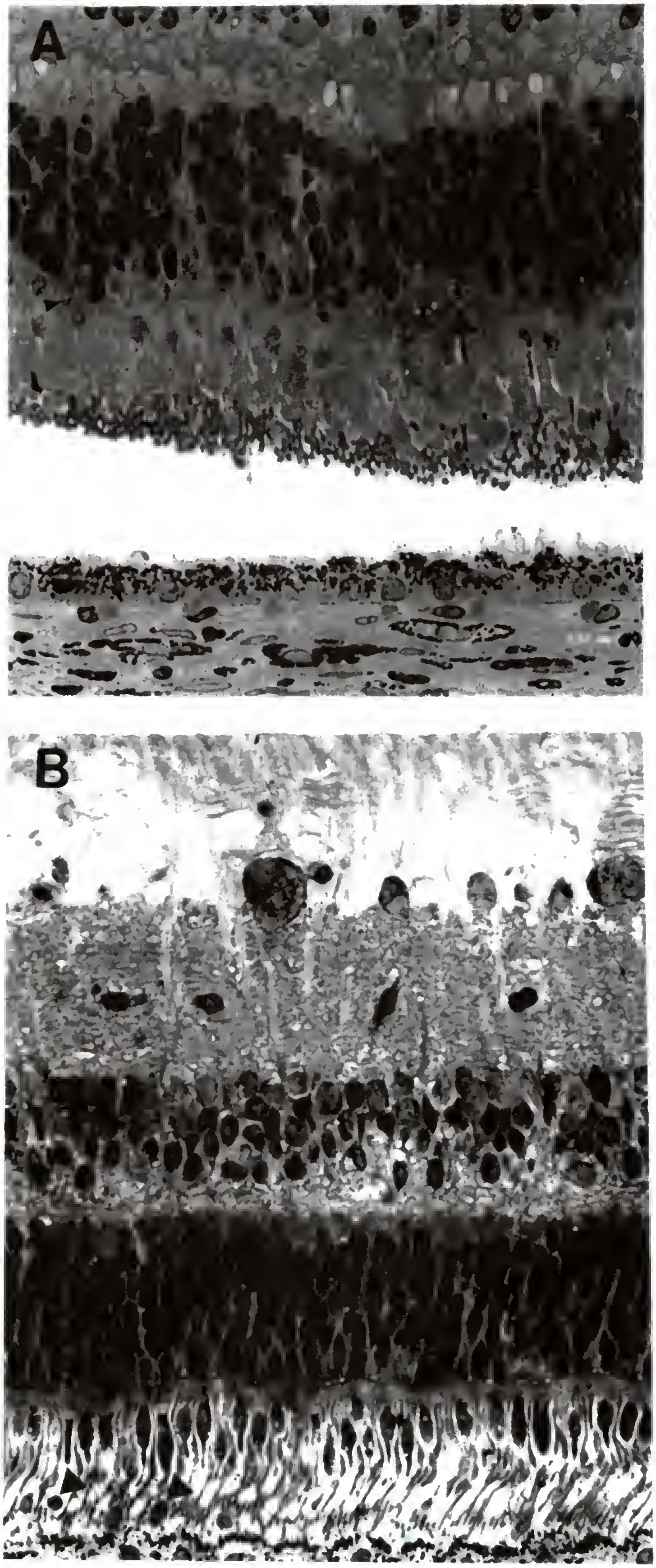
Zinc supplemented diet. The retinas of both eyes of this animal appeared normal. Again, increased intercellular space was apparent in the ONL suggesting loss of nuclei. Also, displaced normal appearing IS were observed on the surface of the RPE. These findings were interpreted as recovery from prior sublethal light-induced-damage (Figure 15b).

Part 1: Intermittent Light Stress (72 hours): 72 hours Post-Exposure (14 days on diet)

Four of the six eyes examined had moderate to severe damage. The animal on the zinc deficient diet had mild damage in one eye and the other was normal. The four damaged eyes were from the animals fed the zinc normal and zinc supplemented diets.

Figure 15: Comparison of retinas of animals fed the ZnN and Zn+ diets. Both animals were exposed to 36 hours of intermittent light and sacrificed 3 weeks post-exposure.

a) Animal on ZnN diet. The retina is artifactually detached but the RPE-OS may have been week as no OS remained adhered to the RPE. There appears to be some loss of nuclei in the ONL layer as intercellular space is increased and cone nuclei are not always on the border of the ELM (arrow). Some IS are vacuolated and within the ONL (arrowheads). The IS and OS region is disorganized but rarely swollen or degenerative suggesting recovery from prior damage may have occurred. The RPE appears normal. (800x) b) Animal fed the Zn+ diet for 3 weeks. Retinal attachment is strong. Several displaced cells and IS (arrowheads) are often apparent in the OS layer on the surface of the RPE. The inner retina is normal. (800x)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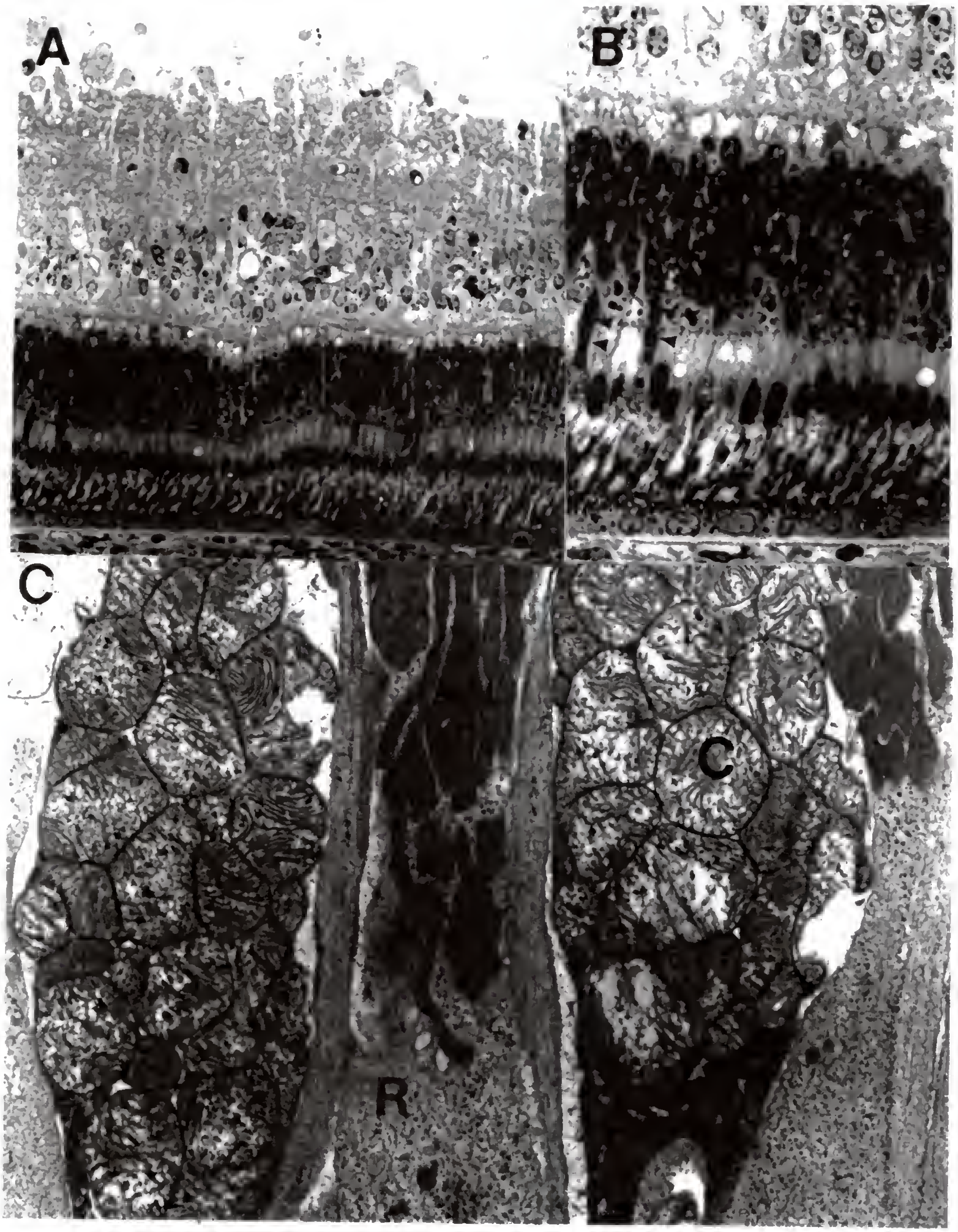
Normal zinc diet. Retinal damage was moderate but limited to outer layers. The left eye had more areas of damage than the right eye. In the right eye the only abnormal change was multifocal pyknosis of nuclei in the ONL, often with displacement of degenerating nuclei through the ELM. The RPE-OS interface was normal. In the left eye, degeneration and displacement of nuclei in the ONL was observed. Disorganization and degeneration of IS and less frequently OS was also present (Figure 16). Ultrastructural examination revealed that most outer segments were normal and they were well embedded in the RPE microvilli (Figure 17). The RPE cells exhibited lighter staining cytosol and a taller profile suggestive of mild intracellular edema. The inner retinal layers were within normal limits. in both eyes. Damage was multifocal, i.e., areas of damage would be found with adjacent areas of relatively normal retina.

Zinc deficient diet. One eye exhibited mild retinal damage. Attachment between the RPE microvilli and outer segments appeared weak as the OS were not well embedded in microvilli (Figure 18). Occasional degeneration and displacement of ONL nuclei through the ELM was observed. Several cone ellipsoids had proximal irregular OS formation. In some areas, mild distal outer segment disorganization was also evident. Some IS were displaced and laying on the surface of the RPE (Figure 19). The other eye of this animal was normal.

Zinc supplemented diet. The retinas of both eyes of this animal were moderately damaged. The right eye exhibited more frequent ONL pyknosis compared to the left eye. Damage was diffuse with edema of all layers. Inner nuclear layer and ONL chromatolysis and pyknosis, disruption and disorganization of the OS, and poor retinal attachment were

Figure 16: Moderate retinal damage in an animal fed the ZnN diet, exposed to 72 hours of intermittent light without heat stress and sacrificed 3 days post-exposure.

a) Multifocal ONL pyknosis and movement through the ELM was observed. The inner retinal layers are normal. (400x) b) Pyknotic nuclei (arrowheads) displacing outward through the ELM. Only mild IS and OS disruption is observed and the RPE is normal. (800x) c) Ultrastructural appearance of degenerating cone IS. Mitochondria are swollen with disintegration of cristae. Note the rod IS are normal in appearance. (10,000x)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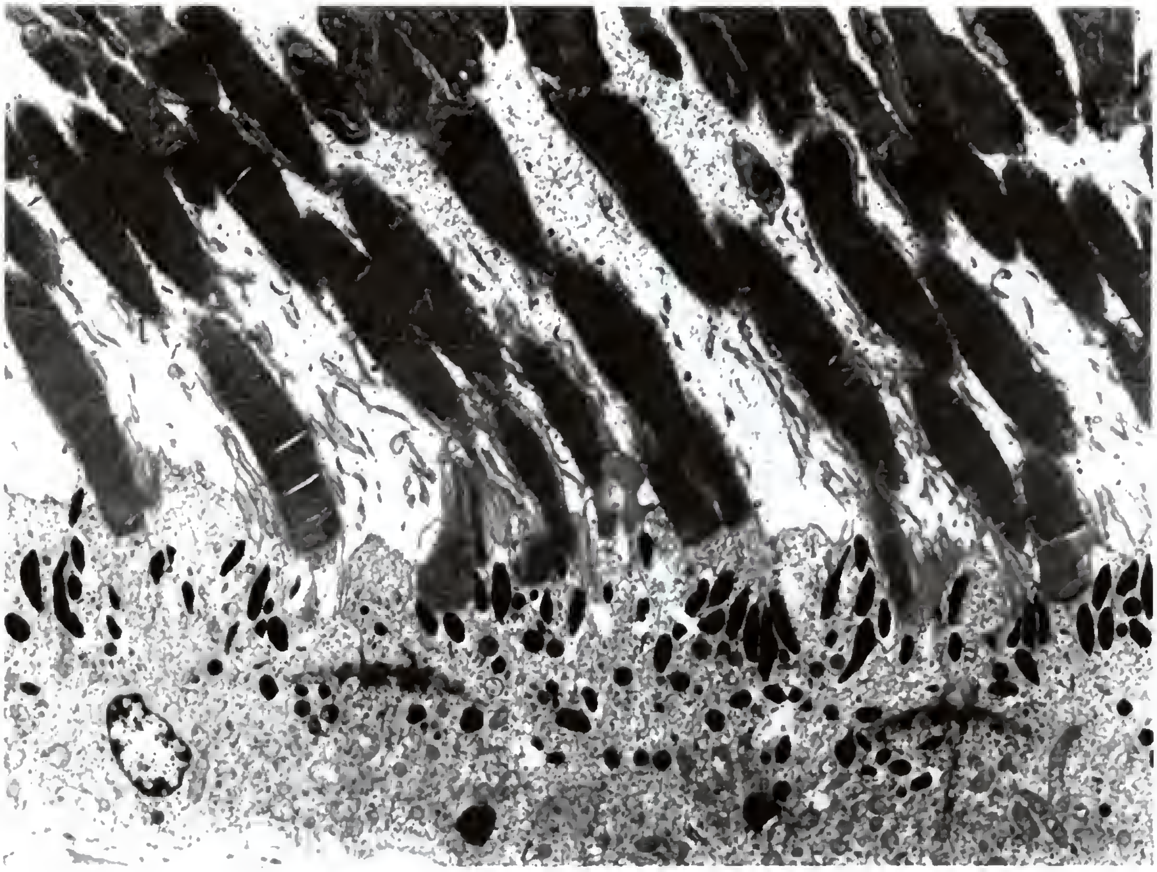

Figure17: Moderate retinal damage in an animal fed the ZnN diet, exposed to 72 hours of intermittent light without heat stress and sacrificed 3 days post-exposure. Same animal as Figure 16. OS are well organized and the RPE-OS interface is intact. The RPE cytosol is lighter staining than the normal porcine RPE (compare to Figure 9) which is suggestive of mild intracellular edema.

Figure 18: Outer retina of animal fed the Zn- diet for 11 days, exposed to 72 hours of intermittent light and sacrificed 3 days post-exposure. Only mild damage was present in one eye of this animal. Detachment of the retina occurred during processing due to poor attachment between the RPE and OS.

a) The outer retina was normal other than the occasional dying nucleus. Subjectively, increased intercellular space is present. (800x) b) The RPE cells are tall and the microvilli elongated. The lack of OS remnants within the microvilli suggest they were not well embedded in the RPE. (2000x)

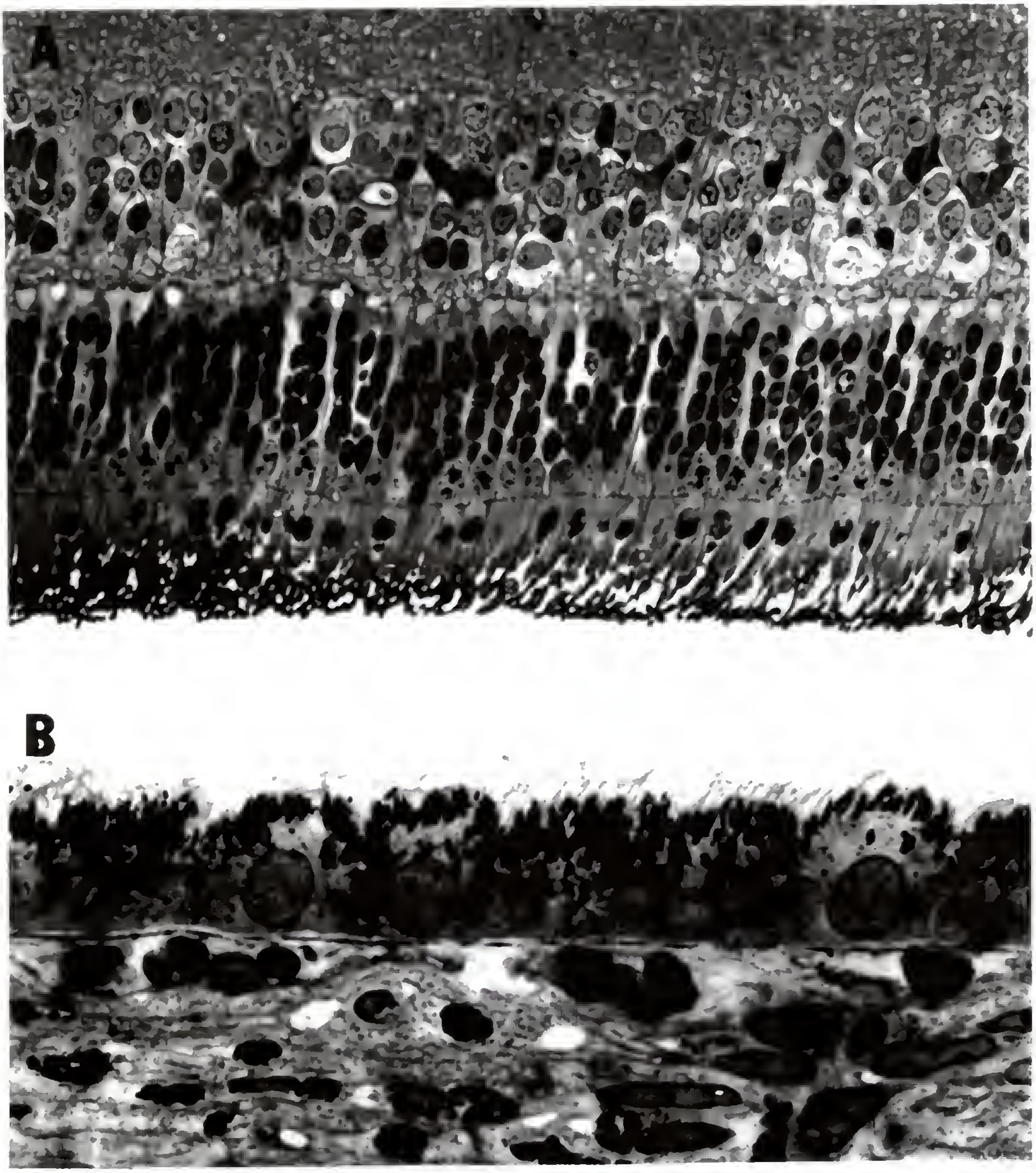
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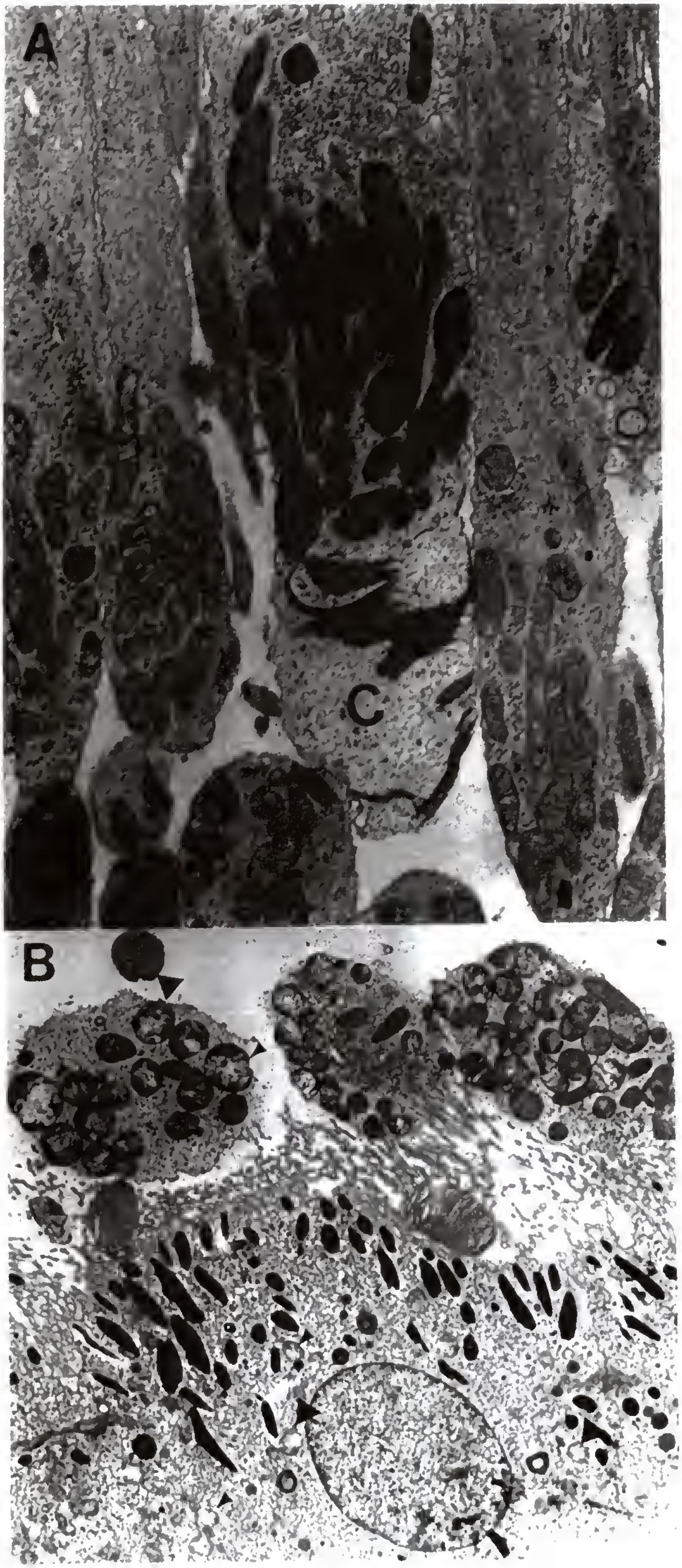
Figure 19: Animal fed the Zn- diet for 11 days, exposed to 72 hours of intermittent light and sacrificed 3 days post-exposure.

a) Abnormal OS formation occurred in the occasional cone photoreceptor (c) but rod and cone IS were otherwise normal. (10,000x) b) Degenerative IS are on the surface of the RPE. The RPE cytosol is less dense than normal RPE suggestive of mild edema. Some mitochondria are swollen (arrowheads). (6000x)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present. Most nuclei in the ONL had lost their typical heterochromatic appearance and many were pyknotic. Degenerating cells were frequently observed moving through the ELM. A few RPE cells appeared edematous (Figure 20).

In the intermittent light stress group, melanin granules in the RPE and choroid appeared normal irregardless of light dose (36 or 72 hours) or diet. Even when RPE edema was present the melanin granules maintained the dense homogeneous and opaque appearance characteristic of mature completely melanized granules (Figures 17 and 19).

Part 1: Intermittent Light Stress (72 hours): 3 weeks Post-Exposure (mean 33 days on diet)

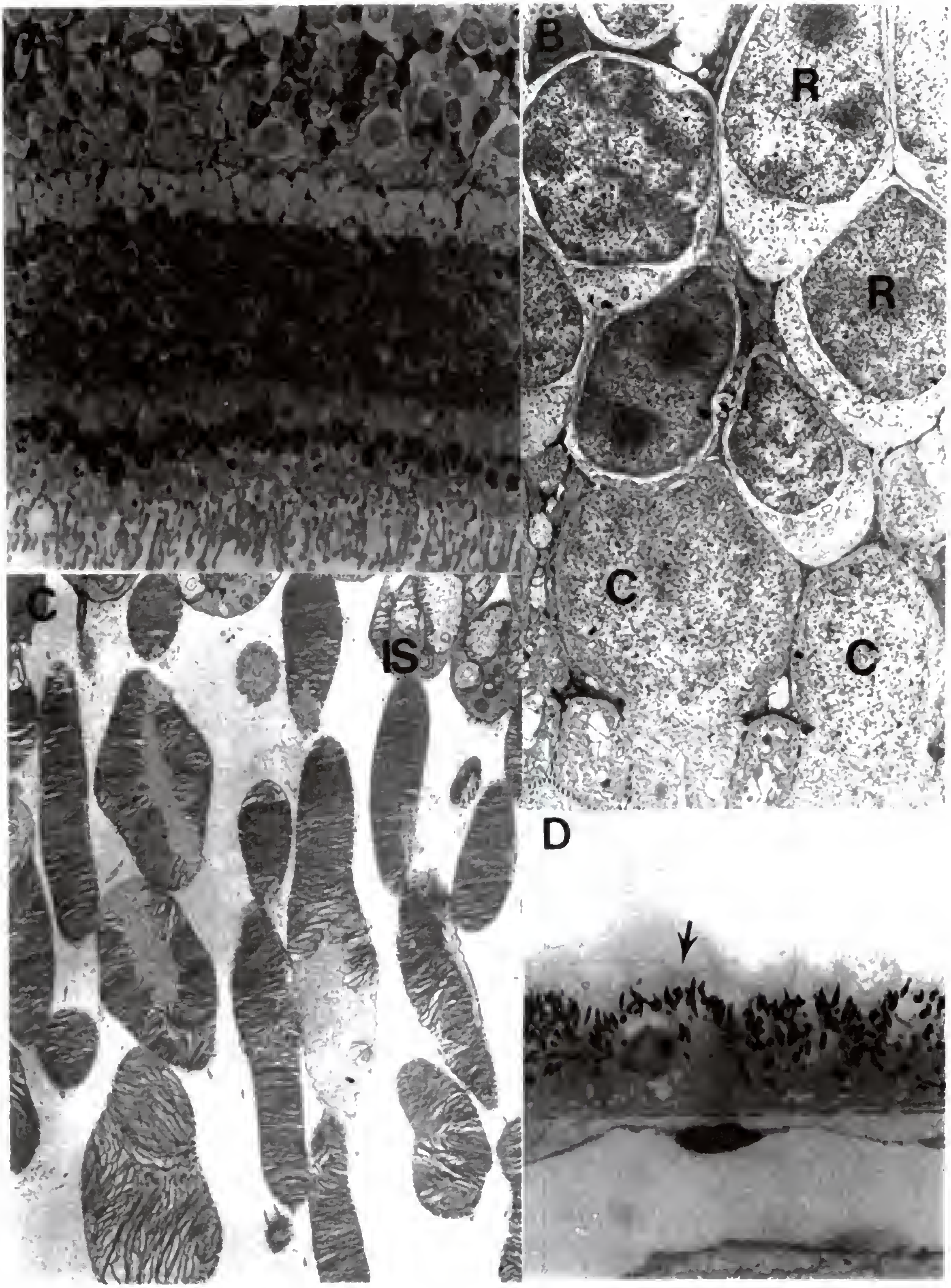
Normal zinc diet. The retinas of both eyes of this animal were normal other than an occasional displaced cell. These cells were displaced through the ELM but had normal nuclear, IS and OS appearance suggesting cellular recovery from prior degeneration.

Zinc deficient diet. Very mild retinal degeneration was present in the left eye; the right eye was normal. In the left eye occasional pyknosis or displacement of ONL nucleus was observed (Figure 21). Recall that occasional pyknosis and displacement of ONL cells occurred in control eyes so whether or not it is significant in this animal is questionable. Outer segments appeared normal and the OS were well embedded in the RPE. Although this animal was on the zinc deficient diet for three weeks and the biochemical data (Table 6) was suggestive of a negative zinc balance, no significant signs of clinical deficiency occurred, i.e., parakeratosis, and the retinas appeared normal.

Zinc supplemented diet. The retinas of this animal were essentially normal. In one eye, there was an area with a few degenerating nuclei moving through the ELM which could represent an area of prior damage.

Figure 20: Retina of animal fed the Zn+ diet for 11 days, exposed to 72 hours of intermittent light and sacrificed 3 days post-exposure.

a) Diffuse edema of all layers is evident. Several INL nuclei are pyknotic. Most ONL nuclei are swollen. The IS and OS are mildly disrupted. The retina is artifactually detached (RPE not shown) due to poor RPE-OS interdigitation. (800x) b) Diffuse ONL chromatolysis of both rods (r) and cones (c) is apparent. (7000x) c) Ultrastructural examination revealed IS and OS disruption and disorganization. d) No OS remnants adhere to the RPE suggesting poor retinal attachment was present. The cell in the center (arrow) exhibits vacuolization and cytosolic edema. (2000x)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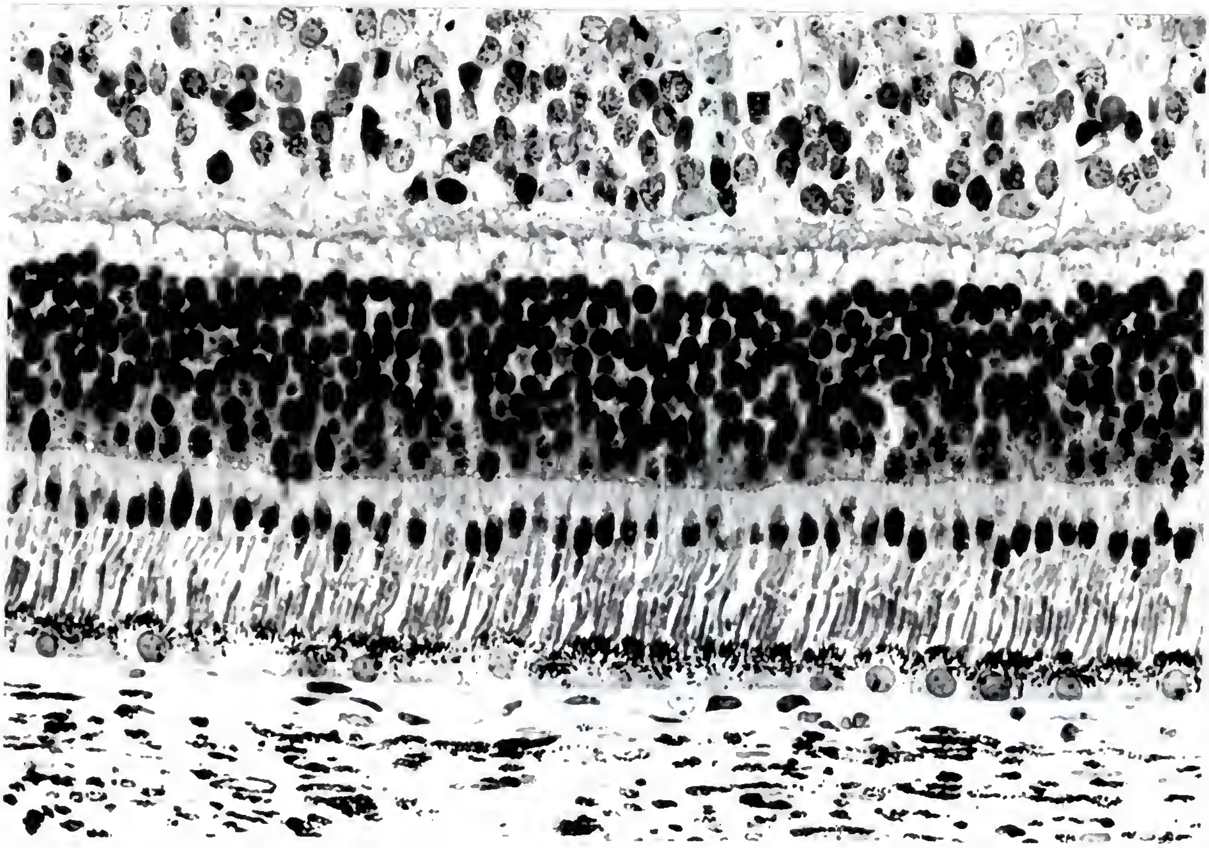

Figure 21: Retina of animal fed the Zn- diet, exposed to 72 hours of intermittent light and sacrificed 3 weeks post-exposure. The retina appears normal. Occasional ONL cells appear degenerative. The RPE-OS interface is normal. (800x)

Part 2: Continuous Light Stress (72 hours); 72 hours Post-Exposure (13 days on diet)

Three animals, 6 eyes, were in each diet group. All received 72 hours of continuous light stress. Sections of central and peripheral retina of the inferotemporal wedge from both eyes were examined. In selected eyes a section from the superotemporal wedge was also examined. In many animals, damage appeared to be multifocal and asymmetric in that one eye was more damaged than the other and within a section there might be a gradient of damage from one end of the section to the other. In animals with severe damage the entire retina, both central and peripheral were degenerative, however some gradient in damage between the two areas often was found. If differences between central and peripheral retina were observed they are specifically discussed. Table 9 summarizes the number of eyes that had normal retinas or various degrees of damage (mild, moderate, or severe). Table 10 utilized more stringent subjective assessment of damage, and sections were designated as having light induced damage only if significant diffuse retinal changes were observed.

Table 9: Summary of qualitative assessment of photic injury in animals exposed to 72 hours of continuous light and sacrificed 72 hours post-exposure.

	Normal	Mild	Moderate	Severe
	# Eyes	# Eyes	# Eyes	# Eyes
Zinc deficient diet (6 eyes)	1	3	2	0
Zinc normal diet (6 eyes)	1	2	0	3
Zinc supplemented diet (6 eyes)	0	3	2	1

Table 10: Light damage in central and peripheral retina of animals exposed to 72 hours of continuous light and sacrificed 72 hours post-exposure. A zero (0), designated no significant damage. Some animals in this group may have had mild degenerative changes but they were not significantly different that changes that were observed in some of the normal eyes. A one, (1), indicates significant retinal degeneration was induced by the light.

Animal number and eye	Diet	Central	Peripheral
178 od	ZnN	1	1
178 os	ZnN	1	1
363 os	ZnN	0	0
363 od	ZnN	0	0
180 os	ZnN	0	0
180 od	ZnN	1	0
179 os	Zn-	0	0
179 od	Zn-	1	0
182 od	Zn-	0	0
182 os	Zn-	0	0
364 od	Zn-	1	1
364 os	Zn-	0	0
365 os	Zn+	0	1
365 od	Zn+	0	0
176 od	Zn+	0	0
176 os	Zn+	0	0
177 os	Zn+	1	1
177 od	Zn+	1	1

Several alterations in melanin in the RPE and choroid were observed in the group exposed to 72 hours of continuous light. Melanin granules in the RPE and choroid often appeared translucent, fragmented, and/ or exhibited a more angular shape (Figure 22). This appearance, which has been observed in the eyes of hamsters exposed to light, may represent a light bleaching effect which may or may not progress to degradation.[183] More striking was the observation of melanogenesis in the RPE of many of the eyes exposed to light-stress. All stages of melanogenesis were observed within the RPE.[184-186] Figure 23 shows several examples of different developmental stages of melanosomes observed in the light-exposed group. Some of the developing melanosomes in this animal appeared to have combined with lysosomes to form melanolysomes. Alternatively, these structures may represent abnormal melanogenesis or possibly melanolipofuscin. The granules in this figure however, most likely represent lysosomes or premelanosomes rather than lipofuscin as the granules are not as osmiophilic as typical lipofuscin granules.[87] Also, the granules shown here are more homogenous in composition and lipofuscin granules usually are more heterogeneous with remnants of outer segments or other undigestible debris.[187,87] Lastly, lipofuscin granules are not very abundant in eyes from animals 8-10 weeks old. The only way to definitively differentiate these granules would be to utilize special stains for lysosomal enzymes such as acid phosphatase. In general, in the light damaged eyes, no great increase in lipofuscin granules was observed compared to control animals that were not exposed to high light intensities.

In many of the RPE cells, large spherical granules with discernible melanofilaments were observed (Figure 23 and 24). With respect to dietary zinc levels, RPE melanogenesis was observed in 5 of 6 eyes in the Zn- group, 4 of 6 eyes in the ZnN group and 5 of 6 eyes in the Zn+ group. Subjectively immature melanosomes were observed much more

Figure 22: Affect of light on melanin granules in the RPE.

a)Animal fed the Zn+ diet for 11 days, exposed to 72 hours of continuous light and sacrificed 3 days post-exposure. The RPE is normal but the melanin granules exhibit a fragmented appearance suggesting alteration of structure due to light treatment. (10,000x) b) Animal fed the Z- diet, exposed to 72 hours of continuous light and sacrificed 3 days post-exposure. Fragmenting melanin granules in the RPE (10,000x)

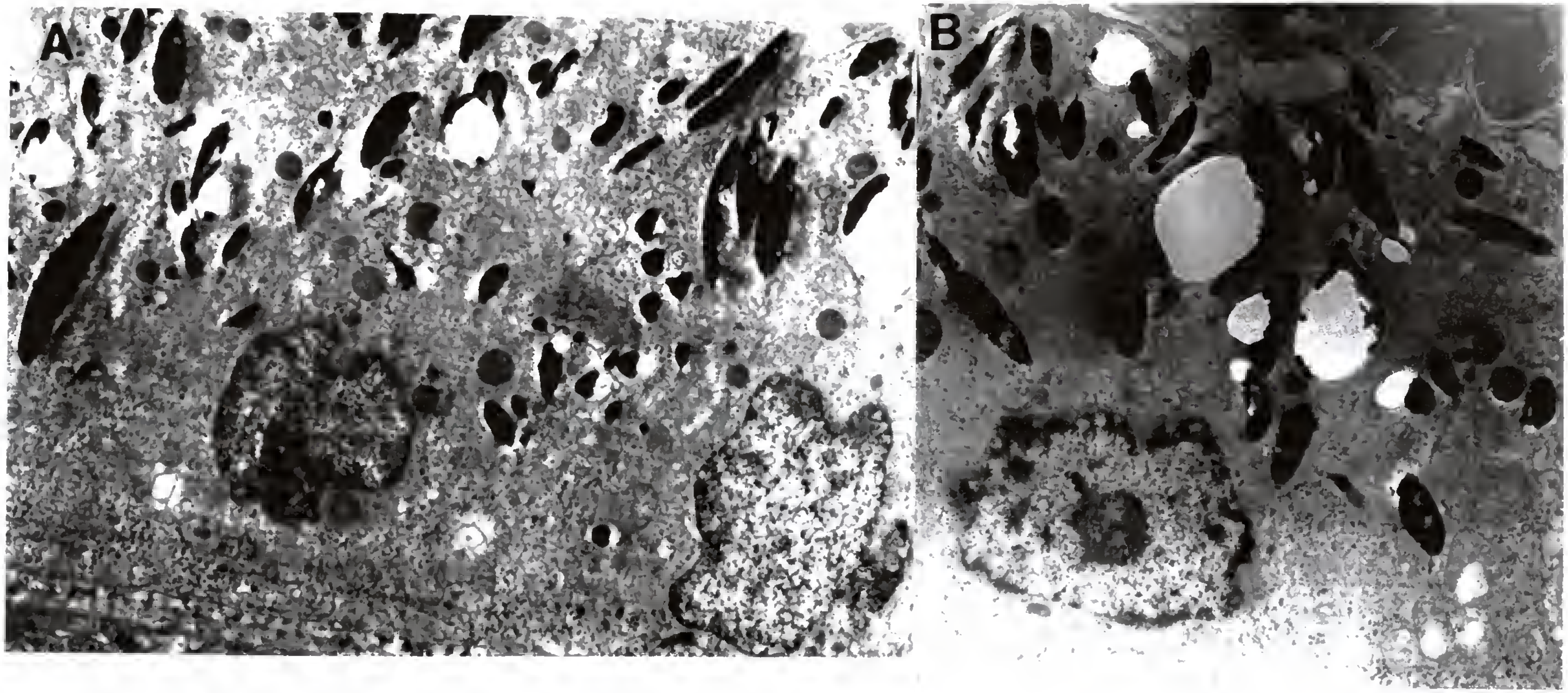
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Figure 23: Melanogenesis was observed in many of the eyes of light-exposed animals.

a) Animal fed the Zn- diet, exposed to 72 hours of continuous light and sacrificed 3 days post-exposure. Increased numbers of granules and melanogenesis was observed in the RPE. Electron micrograph of RPE with increased numbers of granules in the basal and mid cytosol. Several immature melanin granules are present, and some have been engulfed by lysosomes (L) to form melanolysosomes (arrowheads). (8000x) Different developmental stages of melansomes are shown: b) stage III (20,000x), c) stage IV (40,000x) and d) a nearly mature melanosome with dense melanization obscuring all but the peripheral melanofilaments.(30,000x)

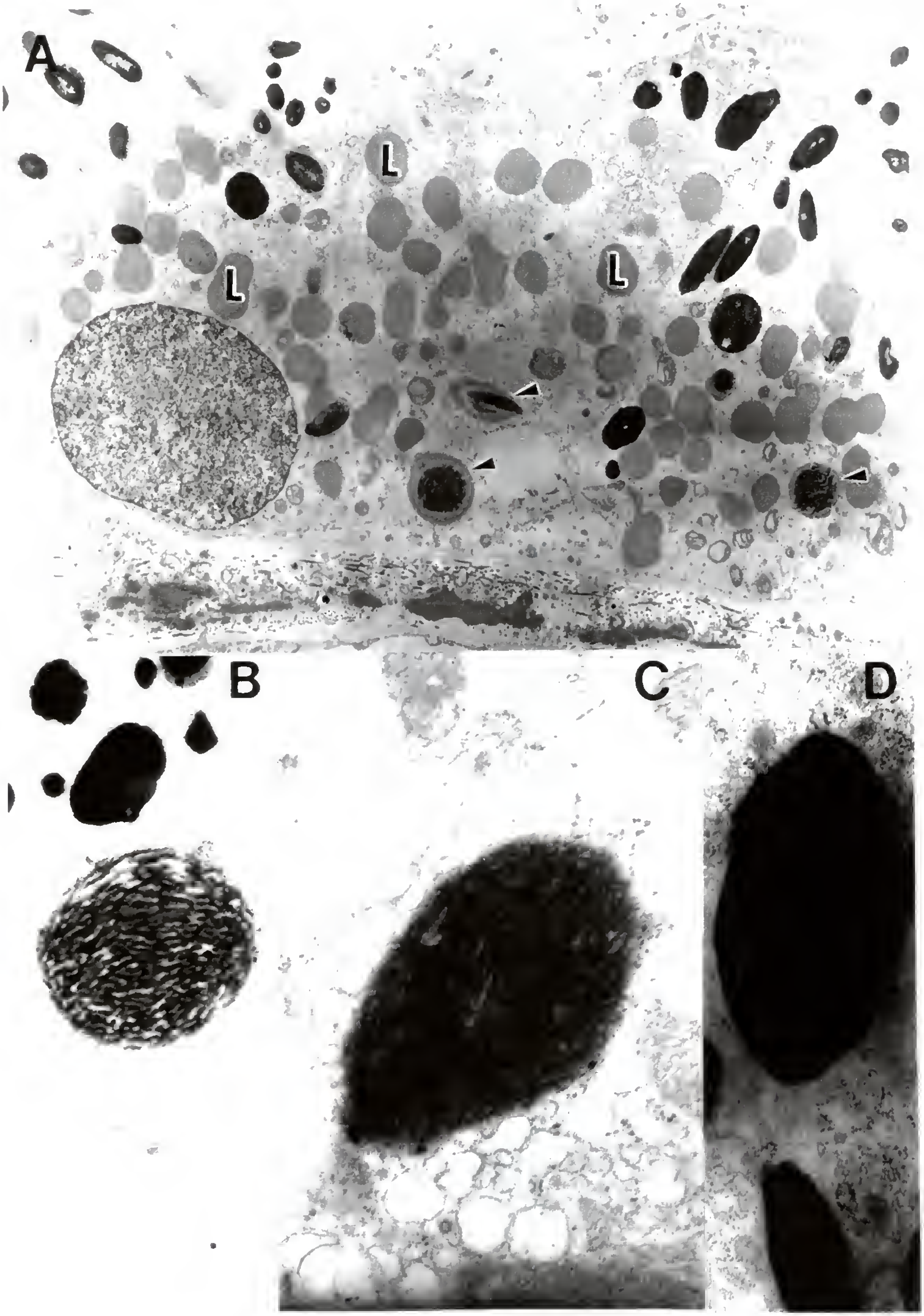
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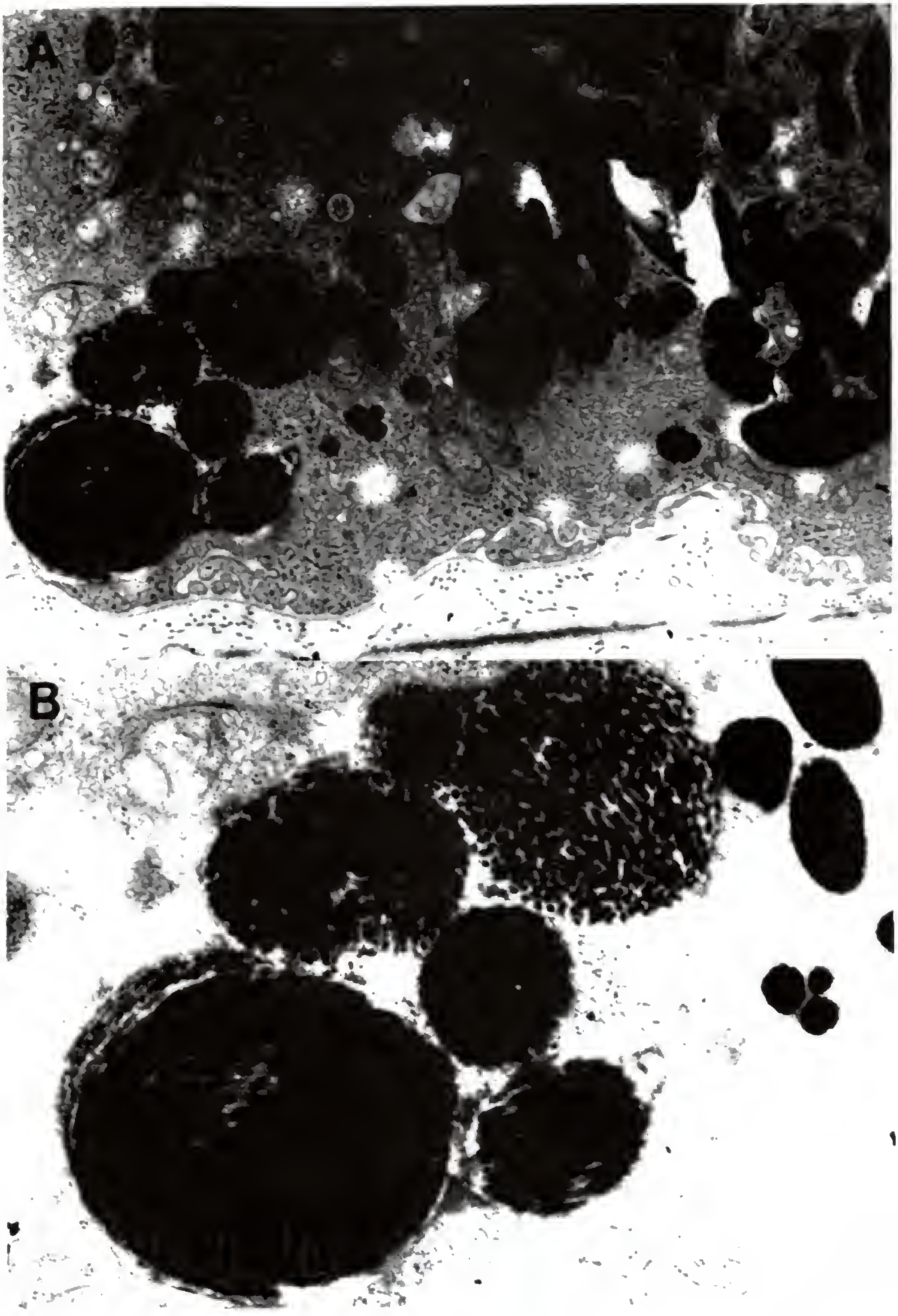
Figure 24: Melanogenesis in light exposed animals. Cluster of immature melanosomes in the RPE of an animal fed the Zn- diet for 11 days and exposed to 72 hours of continous light. Several immature granules are present and most are large and spherical.

a) 14,000x b) 30,000x.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frequently in the eyes of animals fed the Zn- or Zn+ diets. In contrast, melanogenesis was observed only occasionally in control eyes not exposed to light. Evidence of choroidal melanogenesis was observed in only one eye in the animal on the Zn- diet that had been exposed to 72 hours of continuous light (Figure 25).

Zinc normal diet (n=3). Five of the six eyes examined had some degree of damage varying from mild to severe. Three of six eyes or five of the twelve (41.67%) areas examined had significant damage. In one animal one eye was moderately damaged with a gradient from moderate to normal from the central to peripheral retina. Multifocal pyknosis of nuclei in the ONL, and inner and outer segment degeneration were observed. A few INL nuclei were affected. The other eye of this animal was normal.

In the severely damaged eyes, diffuse retinal and RPE edema was present. Nearly all nuclei in the ONL were undergoing pyknosis or other alterations in the nuclear chromatin pattern. Damage was somewhat asymmetric as in the left eye most ONL nuclei were pyknotic and in the right eye all the nuclei of the ONL appeared affected but rather than pyknosis, chromatin alterations suggestive of degeneration were observed. Ultrastructurally, the ONL in the right eye exhibited chromatin densification with maintenance of nuclear shape, while in the left eye severe ONL degeneration and disorganization were observed (Figure 26). In both eyes, RPE damage accompanied the ONL changes (Figure 27). The RPE was severely damaged and few intracellular granules other than melanin could be identified. Inner segment and OS disruption was mild to moderate (Figure 28). Despite normal appearing melanin granules in the RPE of these eyes

Figure 25: Melanin granule changes in choroidal melanocytes were observed in one animal exposed to 72 hours of continous light and fed the Zn- diet.

a) Cluster of small melanosomes in choroidal melanocyte in the choroid.(14,000x)

b) Higher magnification reveals melanofilament profiles within the melanin granules. These may represent melanogenic granules or degradation of granules.(40,000x)

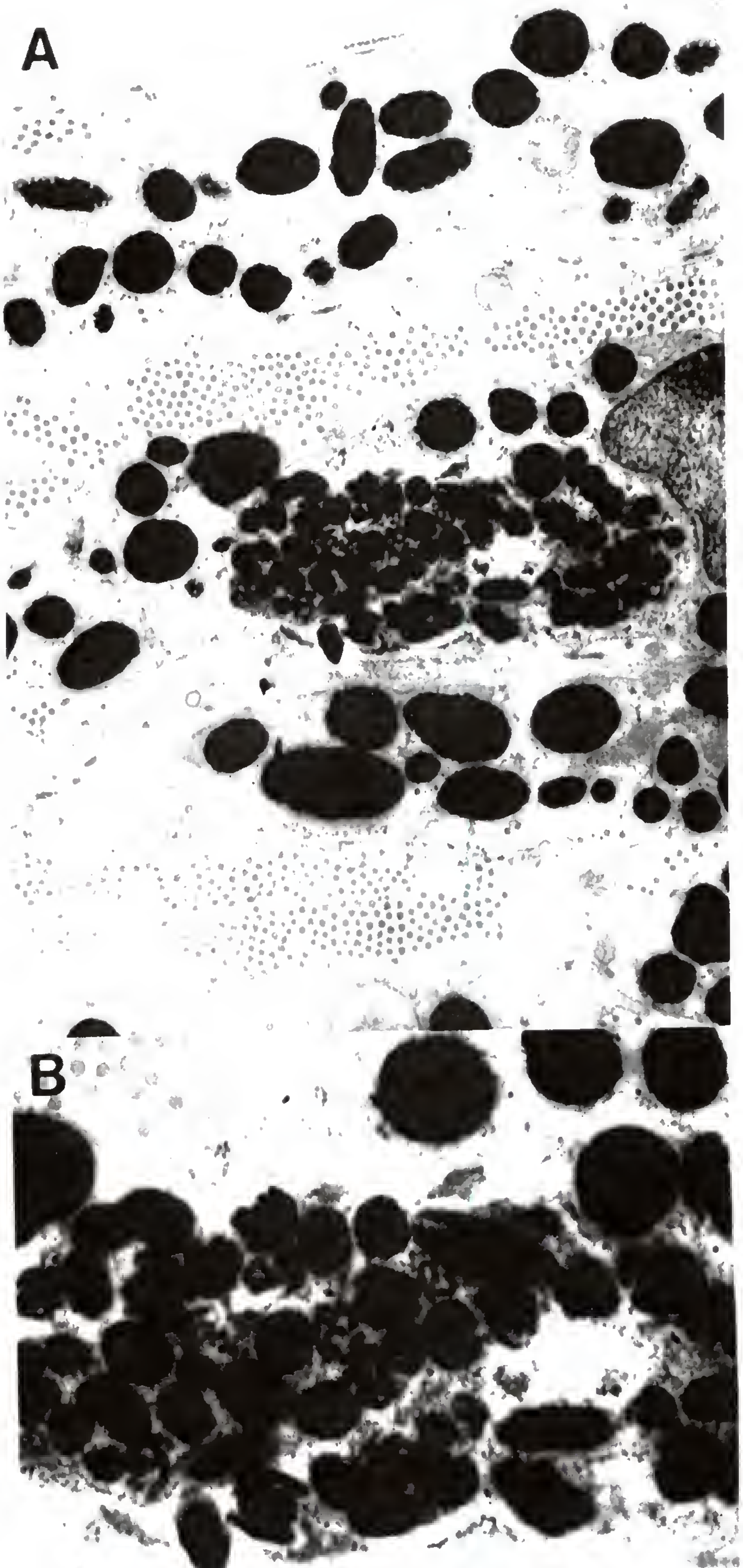
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Figure 26: Animal fed the ZnN diet, exposed to 72 hours of continuous light and sacrificed 3 days post-exposure. a) Diffuse ONL pyknosis was observed in one eye. Several RPE cells are pyknotic and detaching from Bruch's membrane(arrows). (2000x) b) The other eye had less severe changes with ONL chromatin alterations but not frank pyknosis. The RPE however is still severely affected as pyknotic cells are frequently observed (arrows). (2000x) c) ONL nuclear chromatin changes include densification of chromatin suggestive of early pyknosis. (10,000x) d) In the other eye diffuse alteration of ONL is present with pyknosis (p) in some cells and loss of normal nuclear shape. Intracellular edema is present.(7000x)

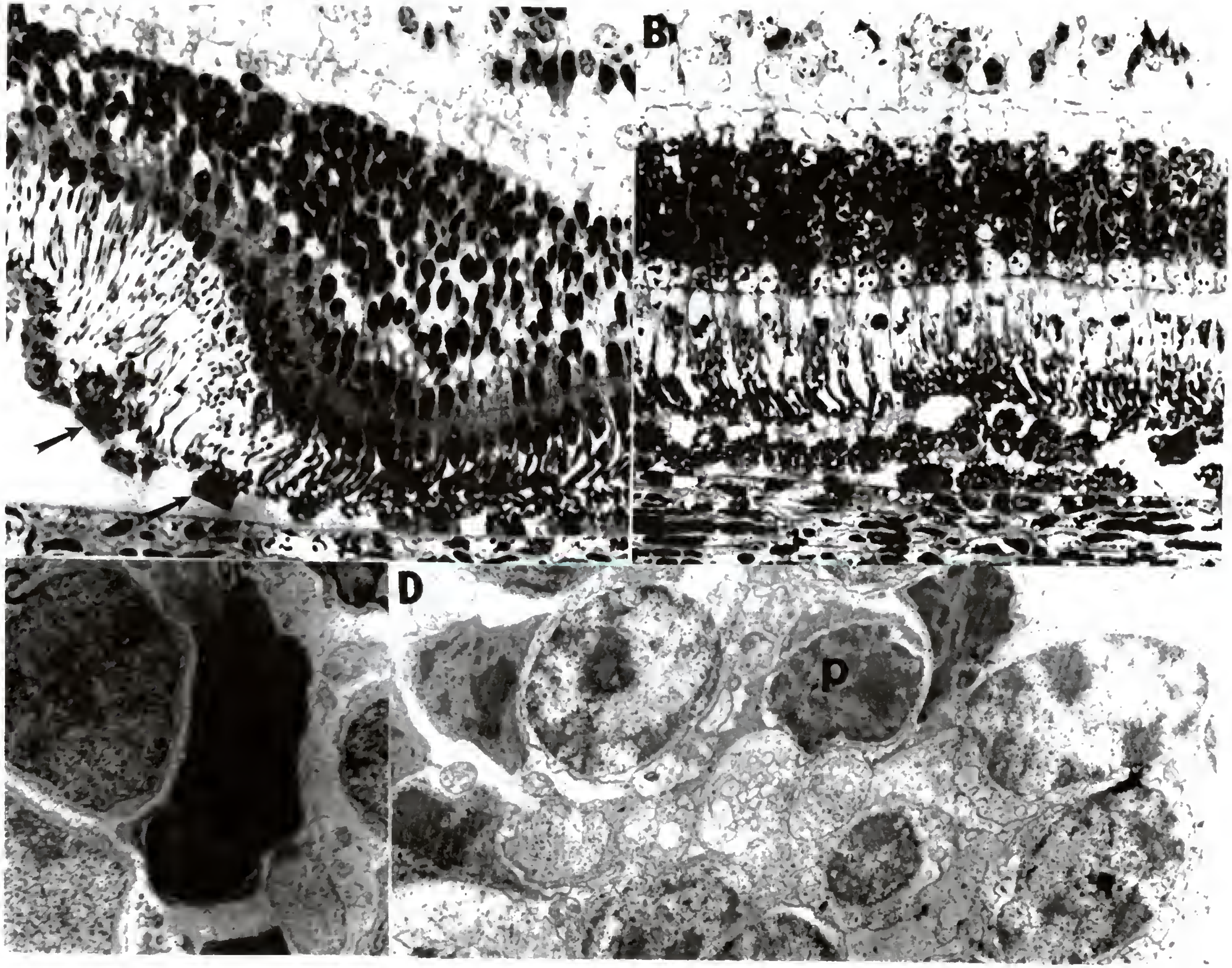
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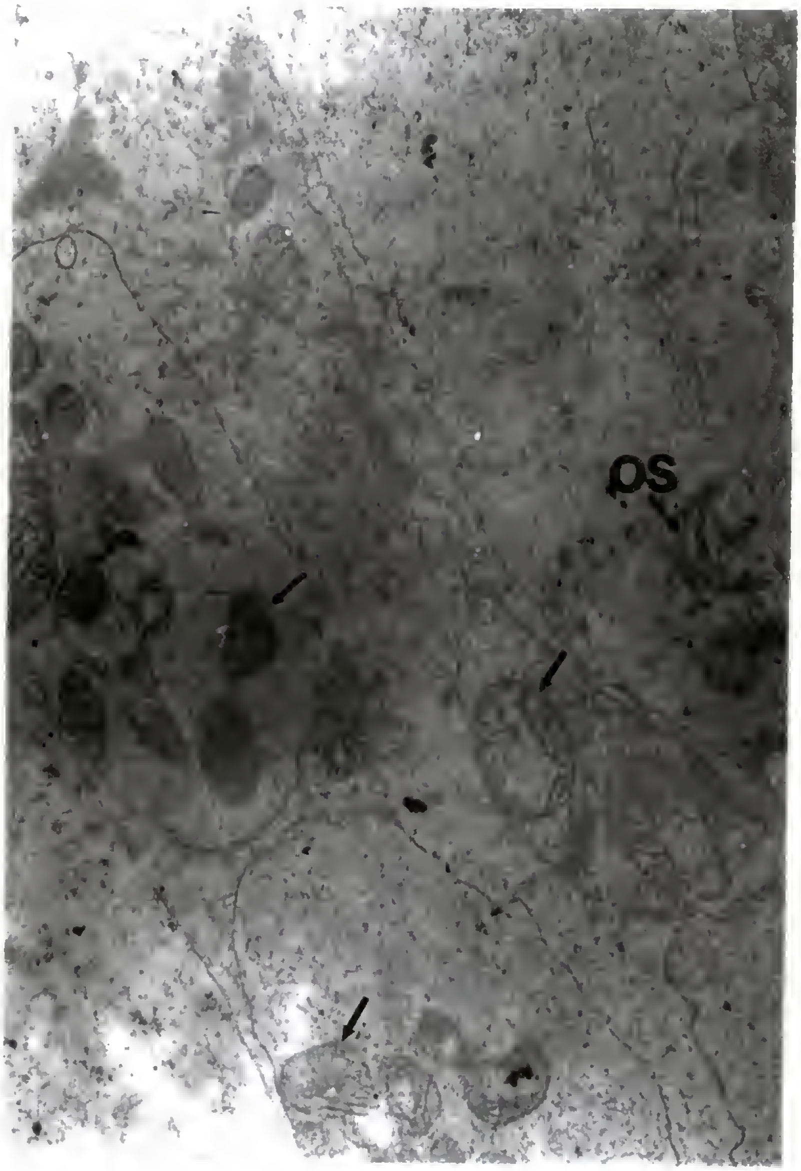
Figure 27: Animal fed the ZnN diet, exposed to 72 hours of continuous light and sacrificed 3 days post-exposure. The RPE was severely damaged and exhibited necrosis and detachment from Bruch's membrane.

a) Detaching RPE cells with debris in interphotoreceptor space. (2000x) b) Ultrastructurally the RPE nuclear chromatin is disintegrating. Melanin granules are normal in appearance. (12,000x)

A
B

Figure 28: Animal fed the ZnN diet, exposed to 72 hours of continuous light and sacrificed 3 days post-exposure. Inner segments are swollen and nearly unrecognizable. Most mitochondria (arrows) have disintegrated and some OS debris is present. Both rods and cones exhibited these changes.(16,000x)


ps

(Figure 27b), choroidal melanocyte granules were translucent and fragmented suggesting disruption of normal granule architecture (Figure 29).

In the third animal of this group mild damage was observed in both eyes. Damage was multifocal in that the retina appeared normal in most areas with foci of damage consisting of a few degenerative (Figure 30). These areas exhibited ONL chromatin changes or pyknosis, IS disruption and the occasional edematous RPE cell (Figure 31). Lipofuscin granules were observed in the RPE but not at numbers significantly above control animals. In these eyes the inner retinal layers were normal.

Zinc deficient diet (n=3). Of six eyes, two eyes appeared normal, two had mild damage and two exhibited moderate damage. Of the twelve areas examined only three (25%) had significant signs of degeneration.

In one animal, retinal damage was asymmetric being mild in one eye and moderate in the opposite eye (Figure 32). Only outer retinal layers were affected. In the moderately damaged eye, numerous nuclei in the ONL were pyknotic and displacing through the ELM. Mild disorganization of outer segments was observed. Damage in the opposite eye was similar except that it was less severe and fewer dying nuclei were observed. The RPE was considered normal: although occasional edematous or degenerative cells were found. A slight increase in abundance of lipofuscin granules compared to control unexposed eyes were observed in the RPE. The melanin in the choroidal melanocytes of this animal appeared fragmented and translucent (Figure 33). The RPE also exhibited fragmentation of many melanin granules.

Figure 29: Animal fed the ZnN diet, exposed to 72 hours of continuous light and sacrificed 3 days post-exposure. Many melanin granules in choroidal melanocytes were abnormal.

a) Several granules were translucent or fragmenting (arrows). (10,000x) b) More normal melanin granules exhibit clumping. (20,000x)

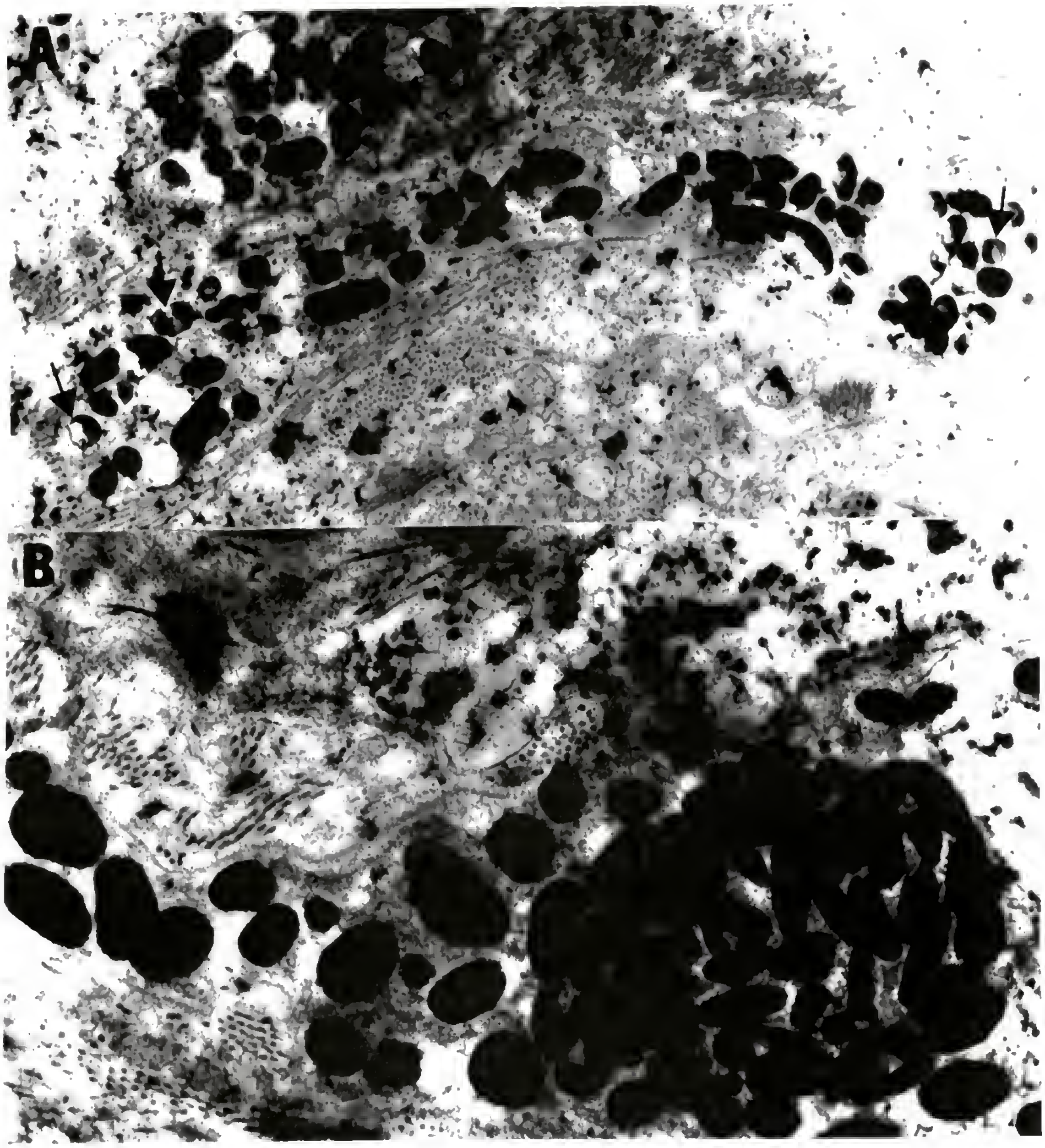

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Figure 30: Animal fed the ZnN diet, exposed to 72 hours of continuous light and sacrificed 3 days post-exposure.

a) Foci of damage with ONL nuclear chromatin changes (arrows)as the only abnormality. (800x) b) Degenerative cone cell (c) with IS mitochondrial swelling and disintegration of the connection between the OS and the ellipsoid. Note the rod (r) IS are normal. (8000x)

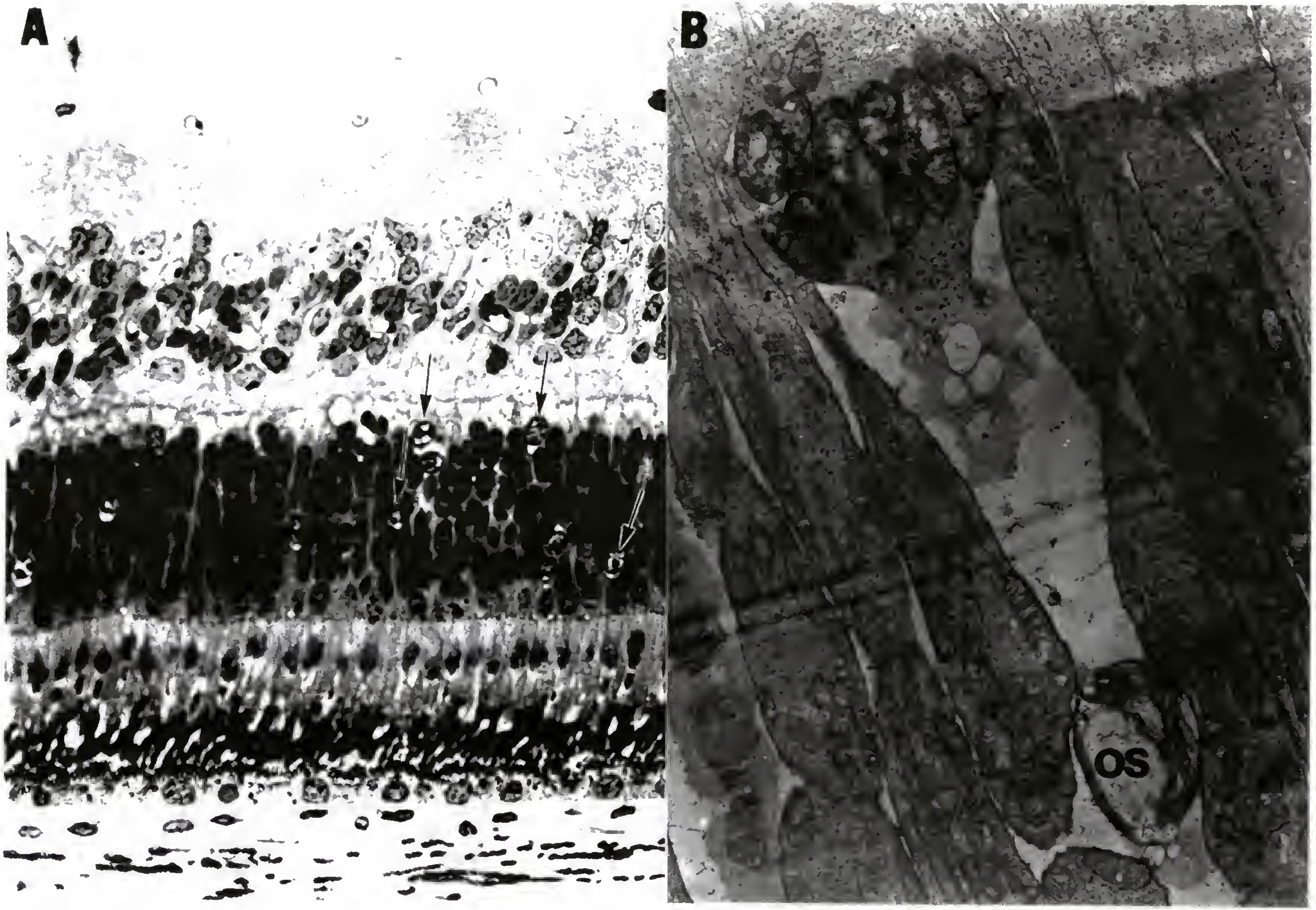
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Figure 31: Animal fed the ZnN diet, exposed to 72 hours of continuous light and sacrificed 3 days post-exposure. The multifocal nature of the damage is apparent when both normal and severe damaged RPE cells can be found in the same thin section. In both cells, however, the melanin granules are fragmenting and translucent, suggesting disruption of normal architecture by light treatment.

a) Relatively normal RPE cell with dense cytosol. The OS are well organized and embedded in the RPE microvilli. (10,000x)

b) Vacuolated and degenerative RPE cell. OS are still well embedded in the apical surface. The cytosol is severely vacuolated and the melanin granules are translucent and fragmenting. (10,000x).

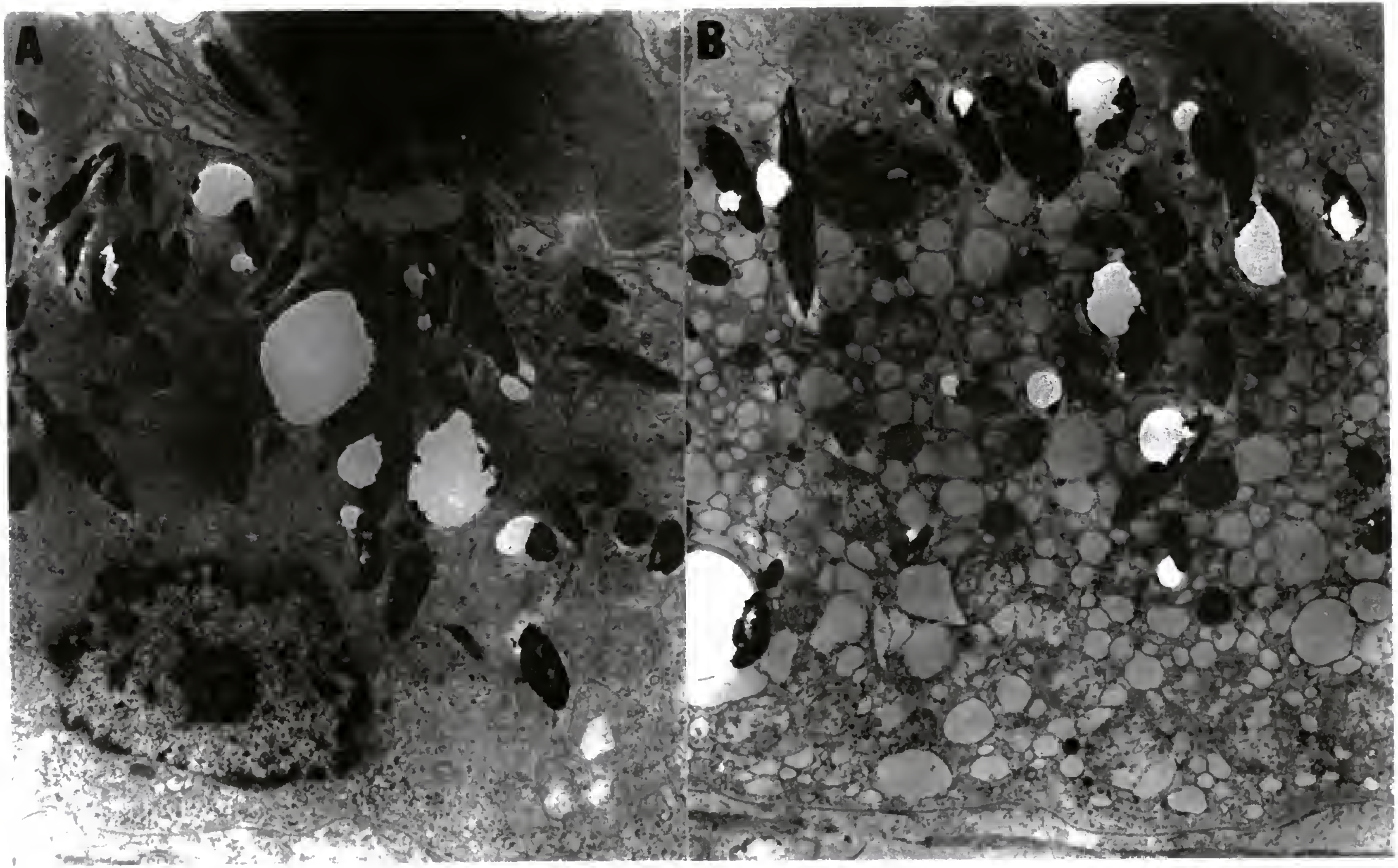
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Figure 32: Animal fed the Zn- diet, exposed to 72 hours of continuous light and sacrificed 3 days post-exposure. Asymmetric damage occurred in this animal.

a) One eye had only mild changes with displacement of some nuclei which did not appear degenerative and a relative increase in intercellular space. The RPE and OS appear normal. (800x). b) The other eye had moderate damage with displacement and degeneration of several nuclei in the ONL. Mild OS and IS disruption was observed. One RPE cell nucleus appears pyknotic (arrow) (800x) c) Again the OS were ressistant to damage as only mild disorganization of OS was present. A clubbed shape OS with disrupted disk lamellae is shown. (40,000x)

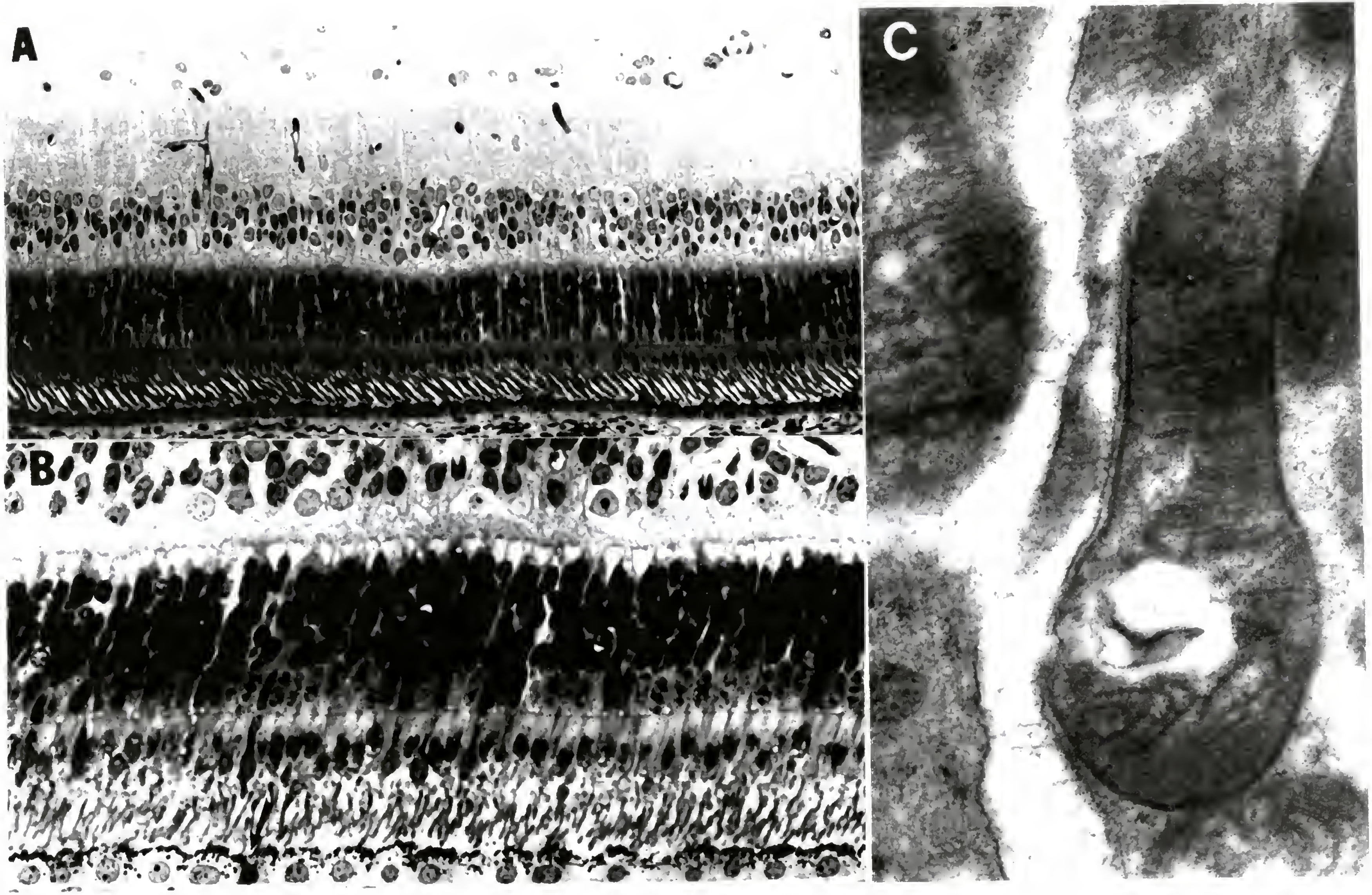
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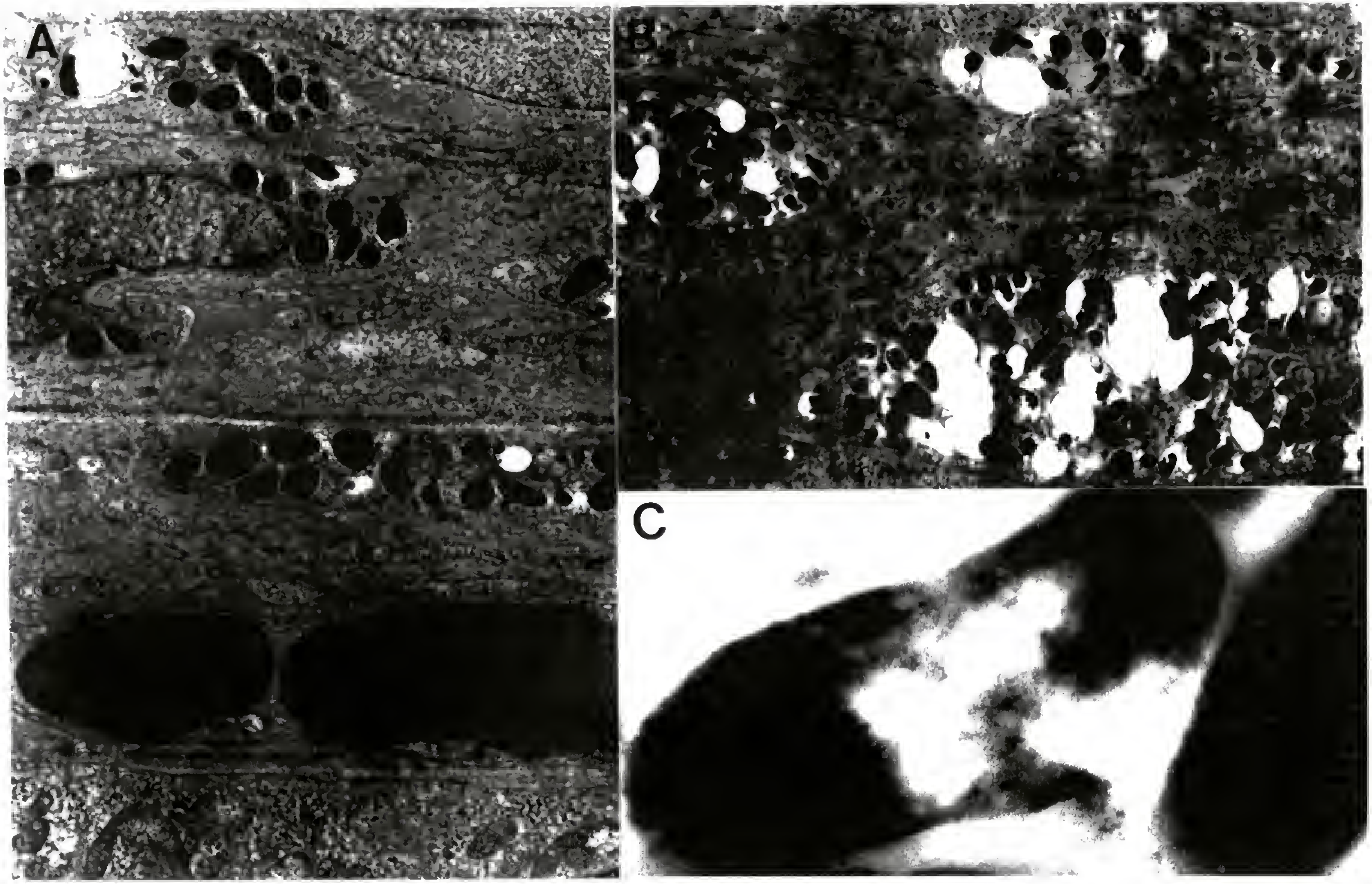
Figure 33: Animal fed the Zn- diet, exposed to 72 hours of continuous light and sacrificed 3 days post-exposure. Choroidal melanocyte melanin granules were disrupted by light treatment.

a) Normal choroid from an control animal on the ZnN diet. b) After light exposure, melanin granules were often fragmented and translucent (arrowheads). (7000x) c) Fragmenting melanin granule. (100,000x)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In another animal, retinal damage was mild in one eye and the other retina was normal. The most significant change was focal degeneration and displacement of a few ONL and the adjacent INL nuclei (Figure 34a). Such an isolated and focal area of degeneration was not considered to be indicative of light induced damage since this was also seen in unexposed controls. RPE and OS in both eyes were normal other than a slight increase in abundance of lipofuscin granules in the RPE compared to control eyes..

The third animal in this group had moderate damage in one eye and the other eye was normal. The outer retina was primarily damaged but mild edema was present throughout the retina (Figure 34b). Abnormal findings included diffuse ONL nuclear changes with pyknosis of rod nuclei and swelling of cone nuclei. Several membrane bound bodies were found in the IS layer suggesting degeneration of IS and some OS. These bodies were near the IS region and the more distal (older) OS were normal which suggested light treatment disrupted normal OS formation. Retinal attachment was poor in the other eye; the OS were so loosely attached and in some areas the microvilli were blunted suggesting in vivo detachment may have been present (Figure 35).

The RPE had several interesting changes in both eyes of this animal (Figure 35). Late stage melanosomes with incompletely melanized melanofilaments were frequently observed. These granules often had a melanizing core with a more translucent periphery. Several melanin granules exhibited a translucent or fragmented appearance. Qualitatively an increase in cytoplasmic granules was observed. It was unclear whether these granules were lysosomes, lipofuscin or premelanosomes, but some exhibited melanin deposition within their centers which suggested they were early stage melanosomes. Some of the

Figure 34: Animal fed the Zn- diet, exposed to 72 hours of continuous light and sacrificed 3 days post-exposure.

a) Infrequent foci of degeneration were observed in one eye. A group of pyknotic cells is found in the INL of this section (arrowheads) but the ONL and RPE-OS are normal. (800x) b) Diffuse ONL pyknosis and intercellular edema is present as well as disruption of the IS, OS and poor retinal attachment. (800x) c) The RPE-OS interface is intact but the membrane bound bodies without any recognizable mitochondria or lamellae are probably remnants of degenerative IS and / or OS. The distal, older, OS (nearer the RPE) are normal suggesting that light treatment disrupted OS formation that occurs proximally at the IS. (10,000x) d) ONL intercellular edema. Rods (r) exhibit pyknosis and cones (c) exhibit chromatolysis. (10,000x)

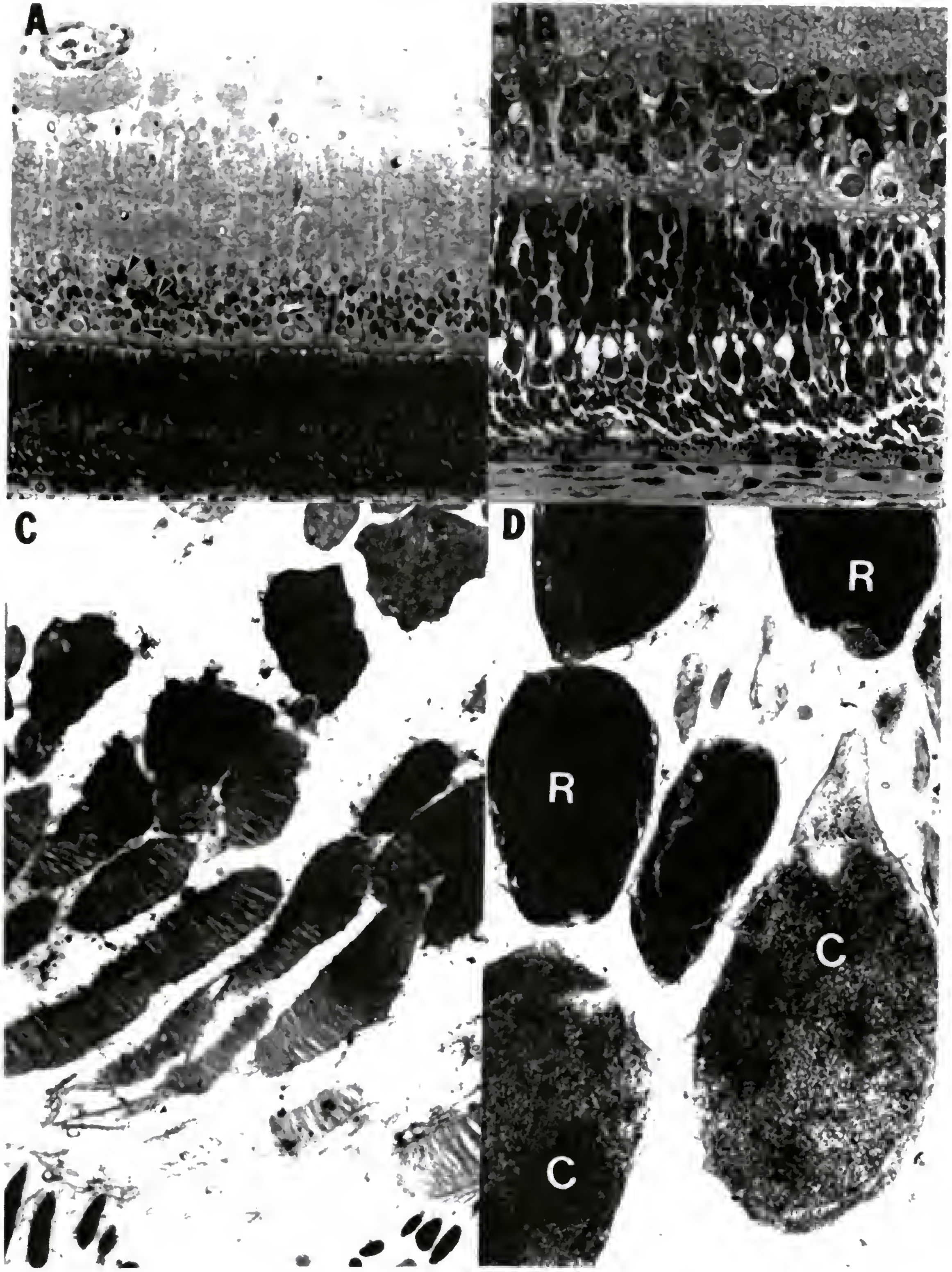
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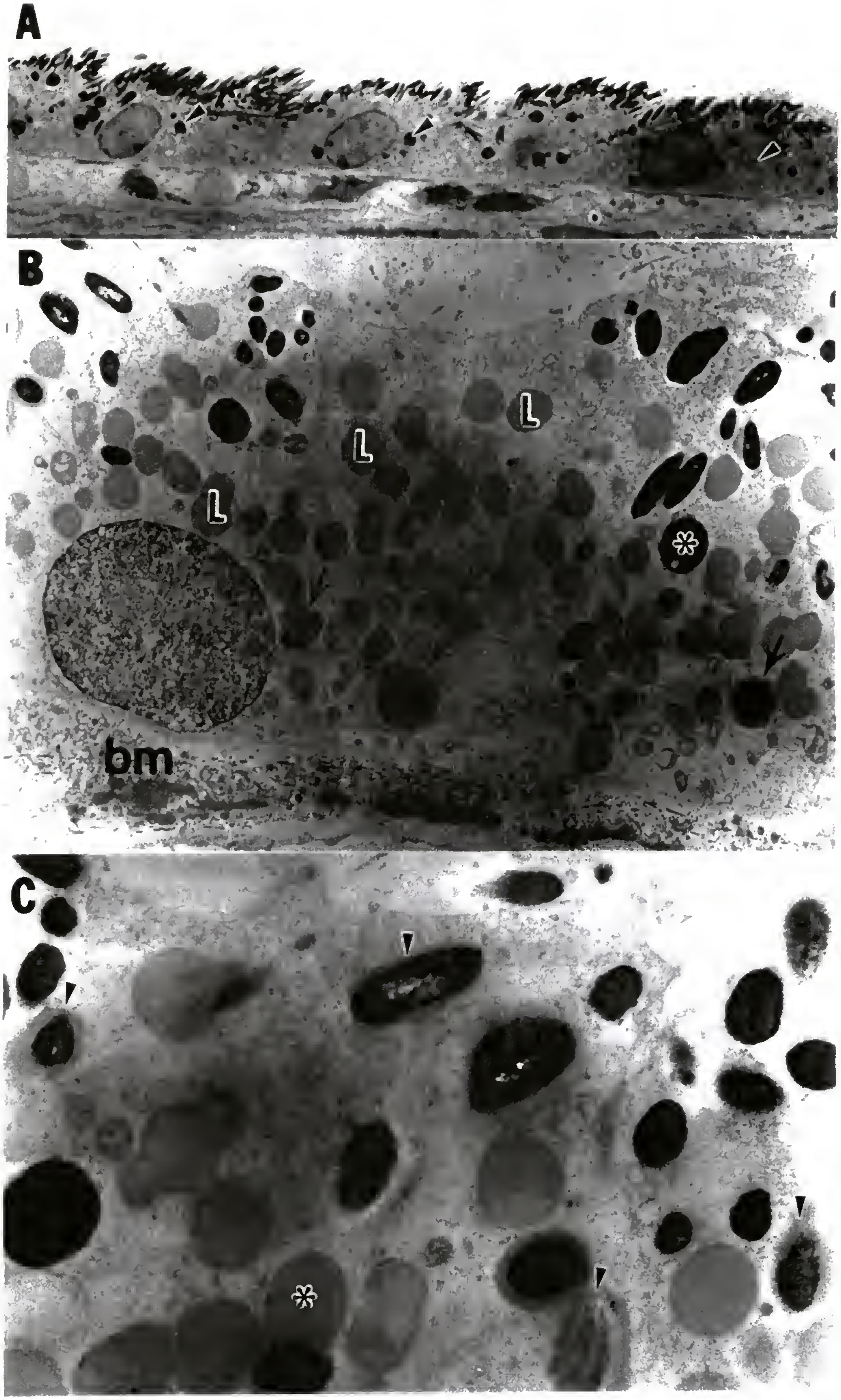
Figure 35: Animal fed the Zn- diet, exposed to 72 hours of continuous light and sacrificed 3 days post-exposure. Increased numbers of granules and melanogenesis was observed in the RPE.

a) Light micrograph of RPE with increased numbers of granules in the basal and mid cytosol (arrowheads). (2000x) b) Electron micrograph of RPE cell with numerous granules in various stages of melanization. Several mature and immature granules (asterisk) have combined with lysosomes (L) to form melanolysosomes (arrows). The RPE microvilli are blunted and poorly developed. No OS are seen because of detachment of the retina. Bruch's membrane (bm) seems to be thickened.(8000x) c) Higher magnification of RPE cytosol with increased granules. Many early stage melanosomes with a smooth surrounding cortex are present (arrowheads). The asterisk denotes a lysosome combining with a mature granule. These complex granules probably represent melanolysosomes in which melanin degradation is occurring. (20,000x)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granules, however, fused with mature melanosomes which was more indicative of them being lysosomal in nature. In Figure 35b, most of the light staining granules are probably lysosomal or premelanosomes as they are less osmiophilic than typical lipofuscin granules. Also there is no evidence of undigested remnants of outer segments or melanin within the limiting membrane. Melanogenesis was observed in 5 of 6 eyes of the pigs fed the Zn- diet and exposed to continuous light for 72 hours.

Zinc supplemented diet (n=3). Damage was moderate to severe in three eyes and mild in three eyes. Of the twelve areas examined five (41.67%) had significant damage. In one animal retinal damage was asymmetric; mild in one eye and moderate in the other. Inner and outer segment disorganization and disruption were observed at the light and electron microscopic level (Figure 36). A moderate number of ONL nuclei were pyknotic. The outer segment-RPE interface was abnormal with notable lack of insertion of outer segments into the RPE microvilli. The RPE had numerous lipofuscin granules. Inner retinal layers were normal. The peripheral retina was less damaged than the central retina. Changes in the opposite eye were similar but less marked.

Both eyes of another animal had mild to moderate damage. In one eye, the damage was multifocal in that only a few areas of retinal degeneration were observed in an otherwise normal retina. In areas of damage, outer and inner segments were disorganized and disrupted with loss of normal architecture. Inner segments could often be found on the surface of the RPE (Figure 36). The occasional RPE cell was degenerating with vacuolated cytosol. Some RPE cells were detaching from Bruch's membrane while nearby cells appeared normal (Figure 37). In severely degenerating RPE cells is what difficult to identify

Figure 36: Animal fed the Zn+ diet for 11 days, exposed to 72 hours of continuous light and sacrificed 3 days post-exposure.

a) Multifocal pyknosis is occurring in the ONL and IS and OS are disorganized. A displaced nucleus is on the surface of the RPE microvilli (arrow). (2000x) b) Damaged areas exhibited IS and OS degeneration. RPE-OS with poor interdigitation between the OS and RPE. OS disorganization is evident (arrowheads) and an IS is within the OS. (10,000x) c) Some membrane bound debris on the surface of the RPE is unrecognizable. An displaced IS with a few remaining mitochondria is next to a large membrane bound body (mb). This may represent IS with some swollen mitochondria that have lost all their cristae. (10,000x).

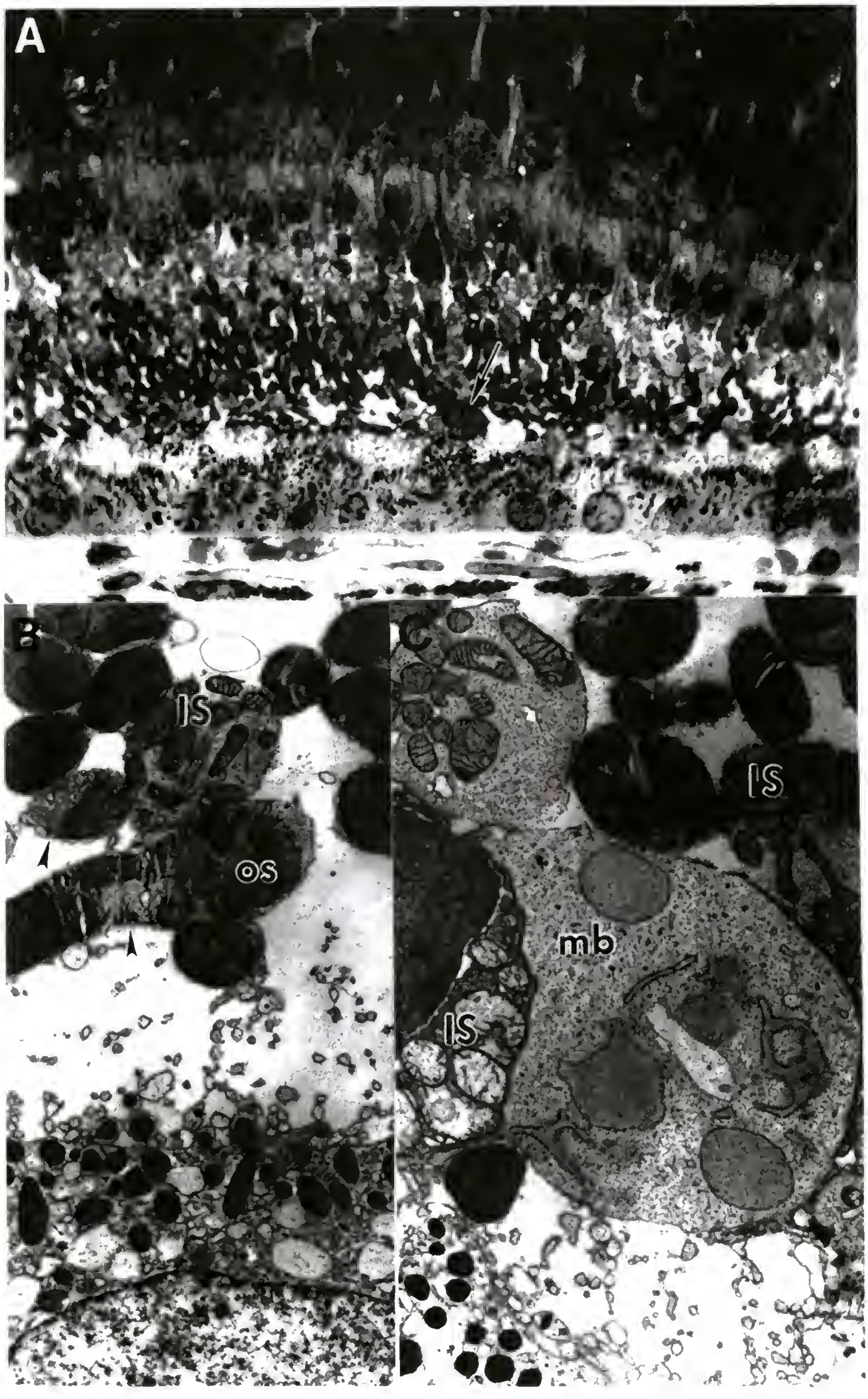
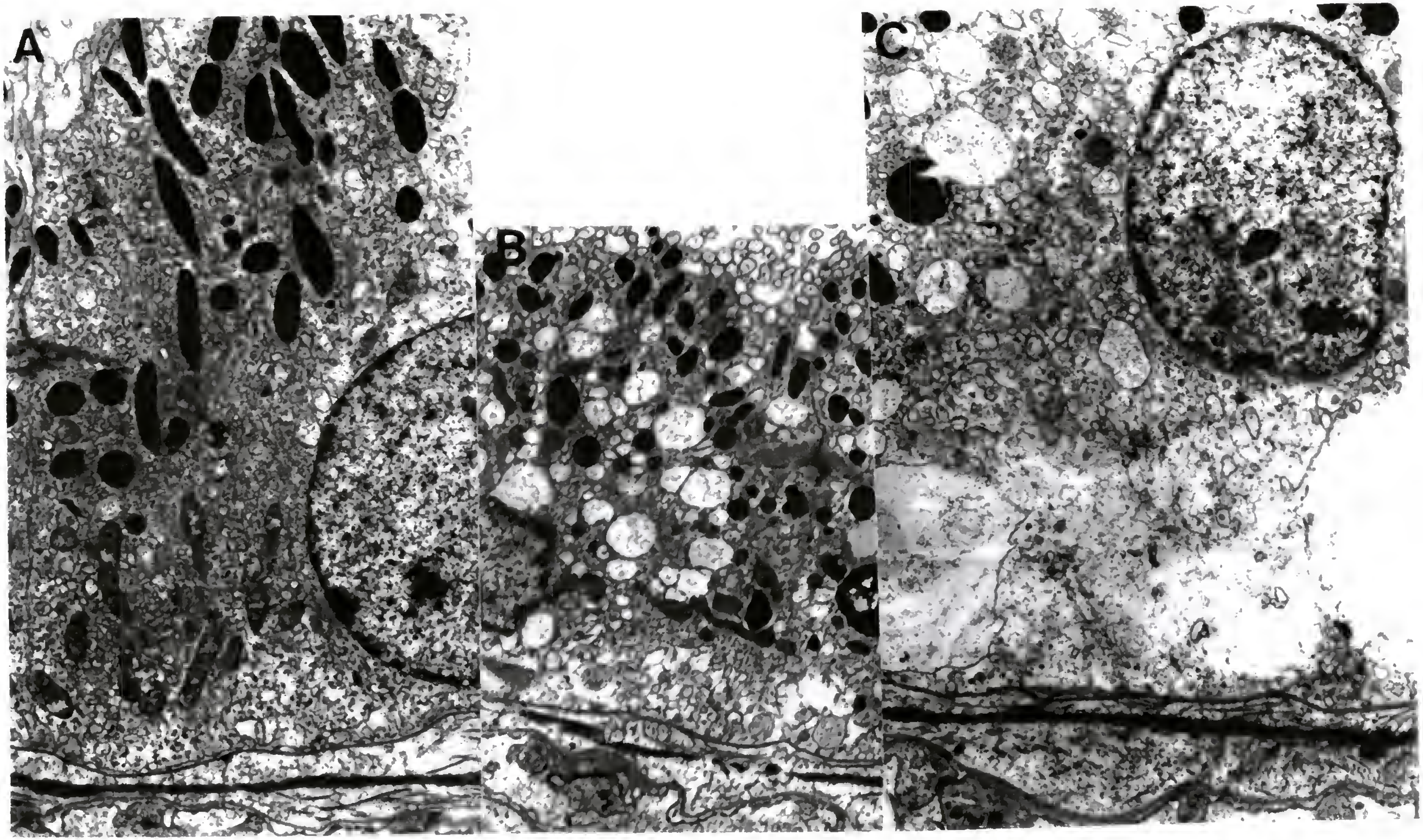

A
B
IS
OS
IS
mb
IS

Figure 37: Animal fed the Zn+ diet for 11 days, exposed to 72 hours of continuous light and sacrificed 3 days post-exposure. RPE changes induced by high dietary zinc and light damage.

a) RPE cell appears normal other than a slight decrease in density of the cytosol which may represent mild edema. (10,000x) b) Degenerative RPE cell with vacuolated cytoplasm. Melanin granules are normal despite marked cellular damage. (8000x) c) RPE cell detaching from Bruch's membrane exhibiting cytosolic edema and vacuolization. Note normal appearing melanin granules. The dark material on the nucleus is precipitated post-staining material. (10,000x)


A
B
C

any intracellular granules other than melanin. In less damaged and normal appearing cells there was a subjective increase in numbers of lipofuscin granules compared to control unexposed eyes.

In one section sub-pigment epithelial neovascularization was observed. A subretinal cell presumed to be a pericyte and an adjacent endothelial cell and lumen, both with their own basal laminae, occurred under the RPE (Figure 38). A nearby area had detaching RPE cells upon which there was grouped nuclei that appeared to be displaced from the ONL (Figure 39). The other eye exhibited similar changes except the ONL had diffuse chromatin changes that varied from pyknosis to the more rounded euchromatic nuclear appearance (Figure 40). Inner retinal layers were normal in both eyes. In damaged cells of the RPE and choroid some melanin granules exhibited a fragmented appearance but this did not always occur (Figure 41).

The third animal in this group had severe damage in one eye and moderate damage in the opposite eye. In one eye severe degeneration of all retinal layers was evident (Figure 42). The RPE cells were necrotic and in some areas the RPE had sloughed off of Bruch's membrane leaving it bare or apposed to ONL cells. Outer and inner segment degeneration was marked. In some areas the ONL was nearly apposed to the RPE and the ELM was difficult to identify. Ultrastructural examination of these areas revealed degenerating RPE cells, adjacent membrane bound debris sometimes recognizable as previously being an outer or inner segment and degenerative photoreceptor nuclei (Figure 43). Within degenerating RPE cells only melanin granules could be identified easily, cytosolic architecture otherwise was severely disrupted. All nuclei in the ONL appeared abnormal with altered chromatin

Figure 38: Animal fed the Zn+ diet for 11 days, exposed to 72 hours of continuous light and sacrificed 3 days post-exposure. Subretinal neovascularizaton.

a) A pericyte (p) and endothelial cell (e) surround a lumen (l) suggesting this is a forming capillary. The overlying RPE cell is vacuolated and contains many granules. A large granule (asterisk) contains melanofilaments and indicates melanogenesis is occurring. (10,000x) b) Higher magnification of pericyte. (24,000x)

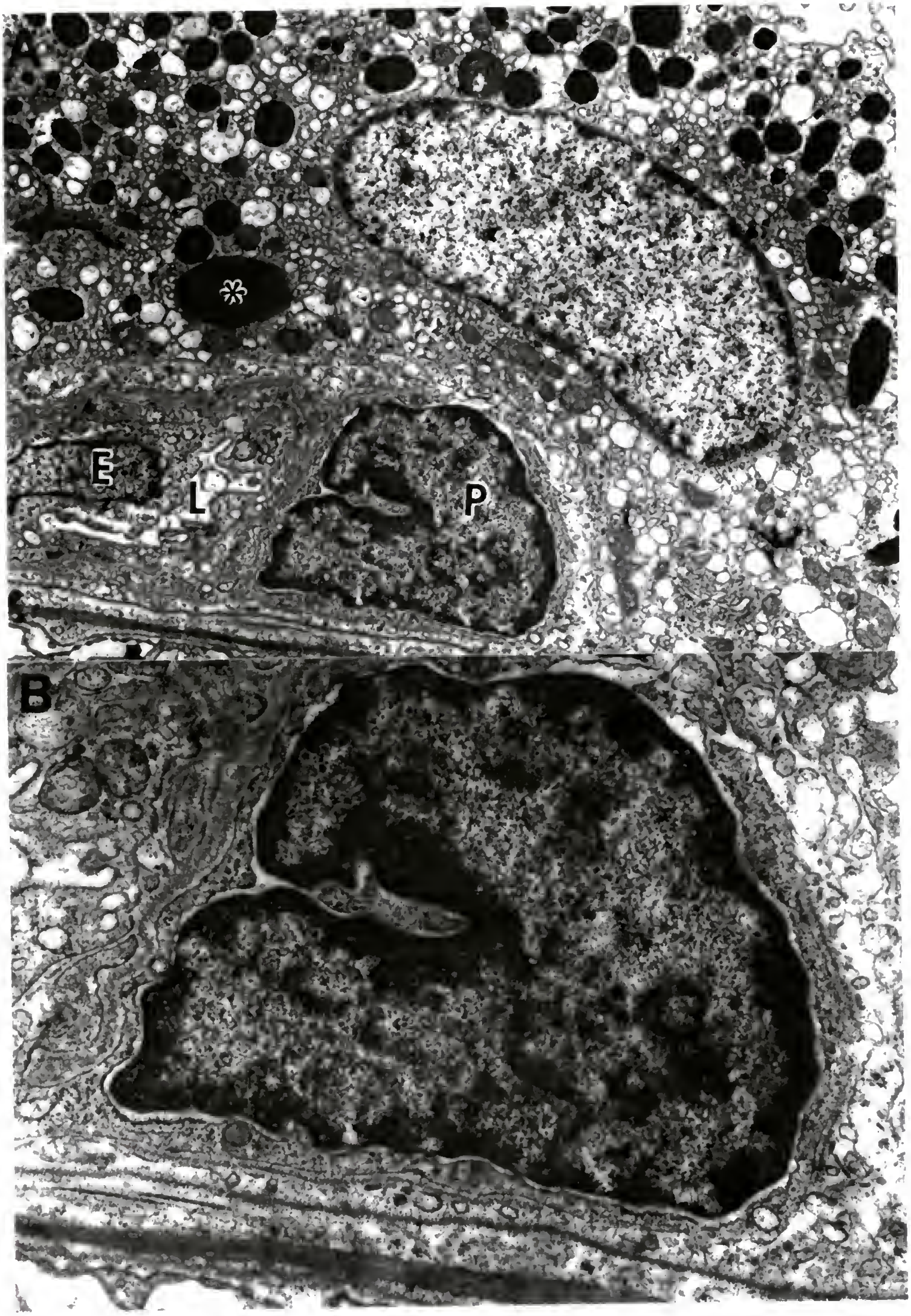

A
E
L
P
B

Figure 39: Animal fed the Zn+ diet for 11 days, exposed to 72 hours of continuous light and sacrificed 3 days post-exposure. A group of ONL cells on the surface of the RPE. The RPE cell was detaching from Bruch's membrane (not shown). Nuclei that resemble ONL rods (r) are on the apical surface of the RPE and the interdigitation between the RPE and these nuclei suggest this did not occur during processing. IS debris is on the surface of these ONL cells and relatively normal OS are found internal to these IS. (10,000x)

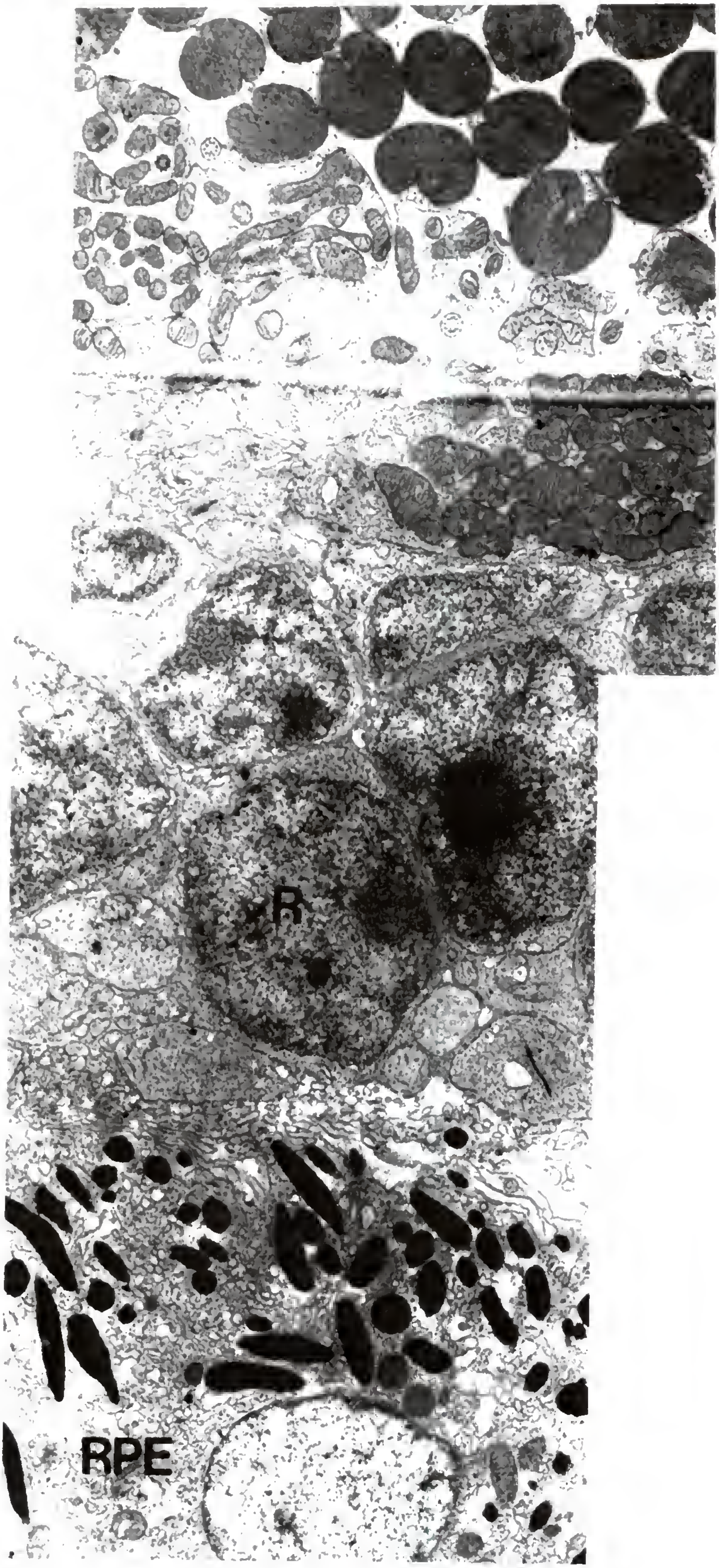

R
RPE

Figure 40: Animal fed the Zn+ diet for 11 days, exposed to 72 hours of continuous light and sacrificed 3 days post-exposure. This is the other eye of the animal shown in Figures 37-39.

a) Diffuse alteration of ONL nuclear chromatin pattern is apparent. Nuclear chromatin densification and increased euchromatin with rounding of cells is present. Several cone pedicles (arrowheads) have swollen mitochondria. (2000x) b) Vacuolization and detachment of the RPE is occurring.(2000x) c) Not all RPE cells were abnormal. The RPE cell on the right is relatively normal while that on the left is severely vacuolated. (10,000x)

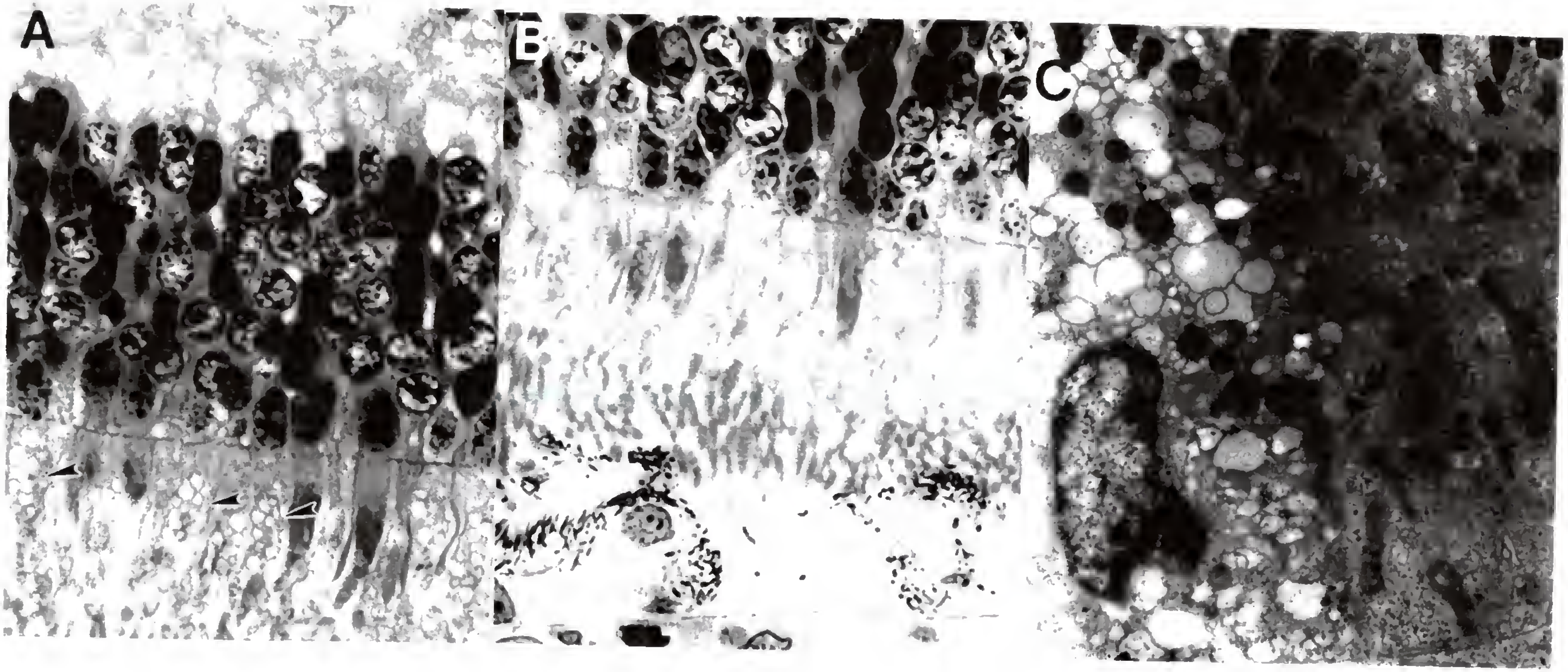

A
B
C

Figure 41: Animal fed the Zn+ diet for 11 days, exposed to 72 hours of continuous light and sacrificed 3 days post-exposure. Melanin granules often exhibit a fragmented appearance suggesting alteration of structure due to light treatment.

a) Relatively normal RPE cell with fragmenting melanin. (10,000x) b) Choroidal melanocyte granules with less frequent fragmentation. (24,000x)

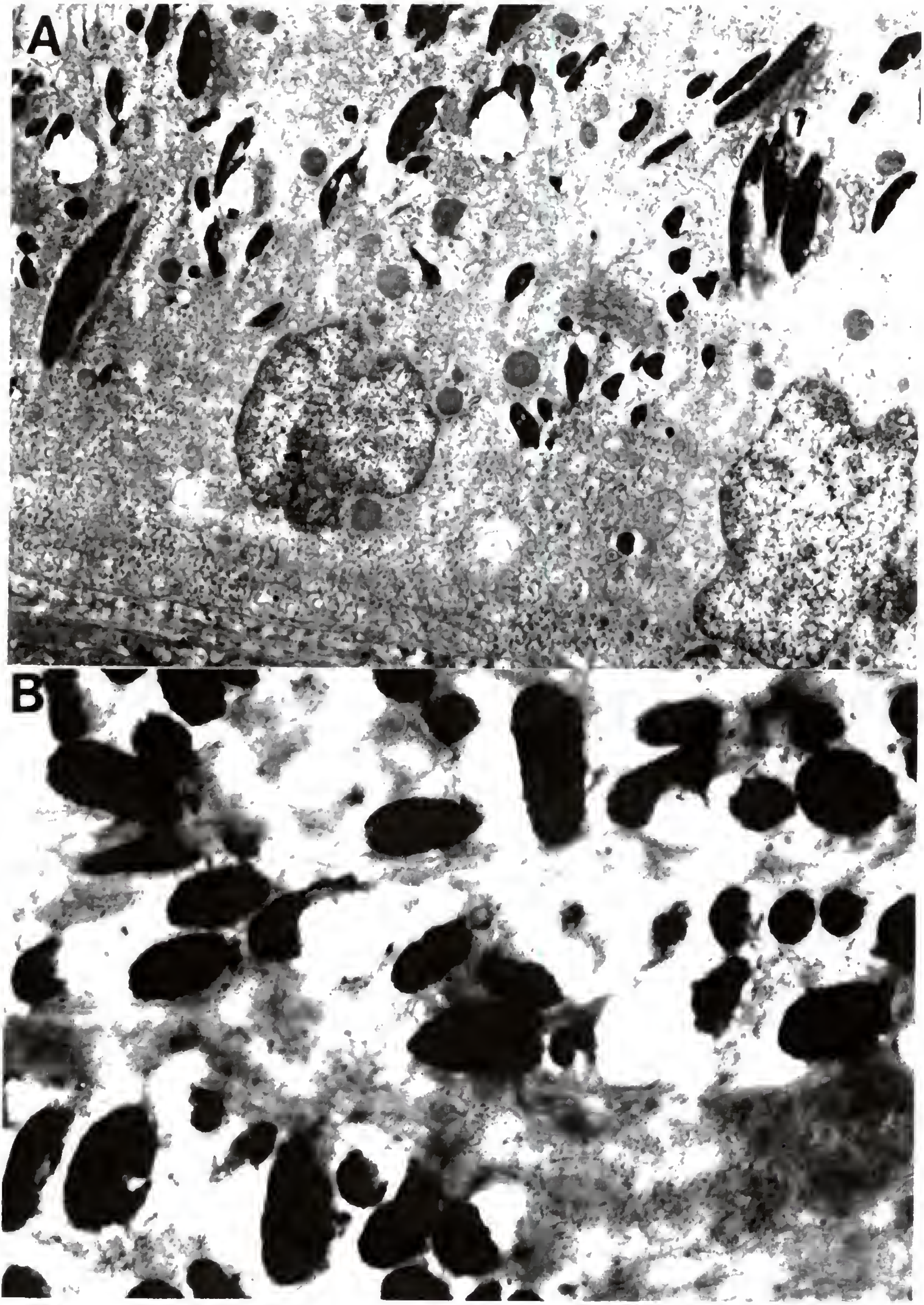

A
B

Figure 42: Animal fed the Zn+ diet for 11 days, exposed to 72 hours of continuous light and sacrificed 3 days post-exposure. Severe diffuse retinal damage.

a) Diffuse retinal edema is present. RPE has sloughed off of Bruch's membrane (arrowheads) in several areas (arrowheads). INL and ONL pyknosis is occurring. The ONL layer is significantly thinned and absent in some areas.(400x) b) Note detaching RPE cells, pyknotic ONL nuclei and total disruption of RPE layer and ELM. (2000x) c) Higher magnification of areas with necrotic RPE cells detaching from RPE and pyknotic ONL nuclei nearly apposed to the RPE. (2000x) d) Area of bare Bruch's membrane with OS on its surface. Note that most of the IS that have survived are cones (c). Swollen horizontal cells (asterisk) are seen in the INL. (2000x)

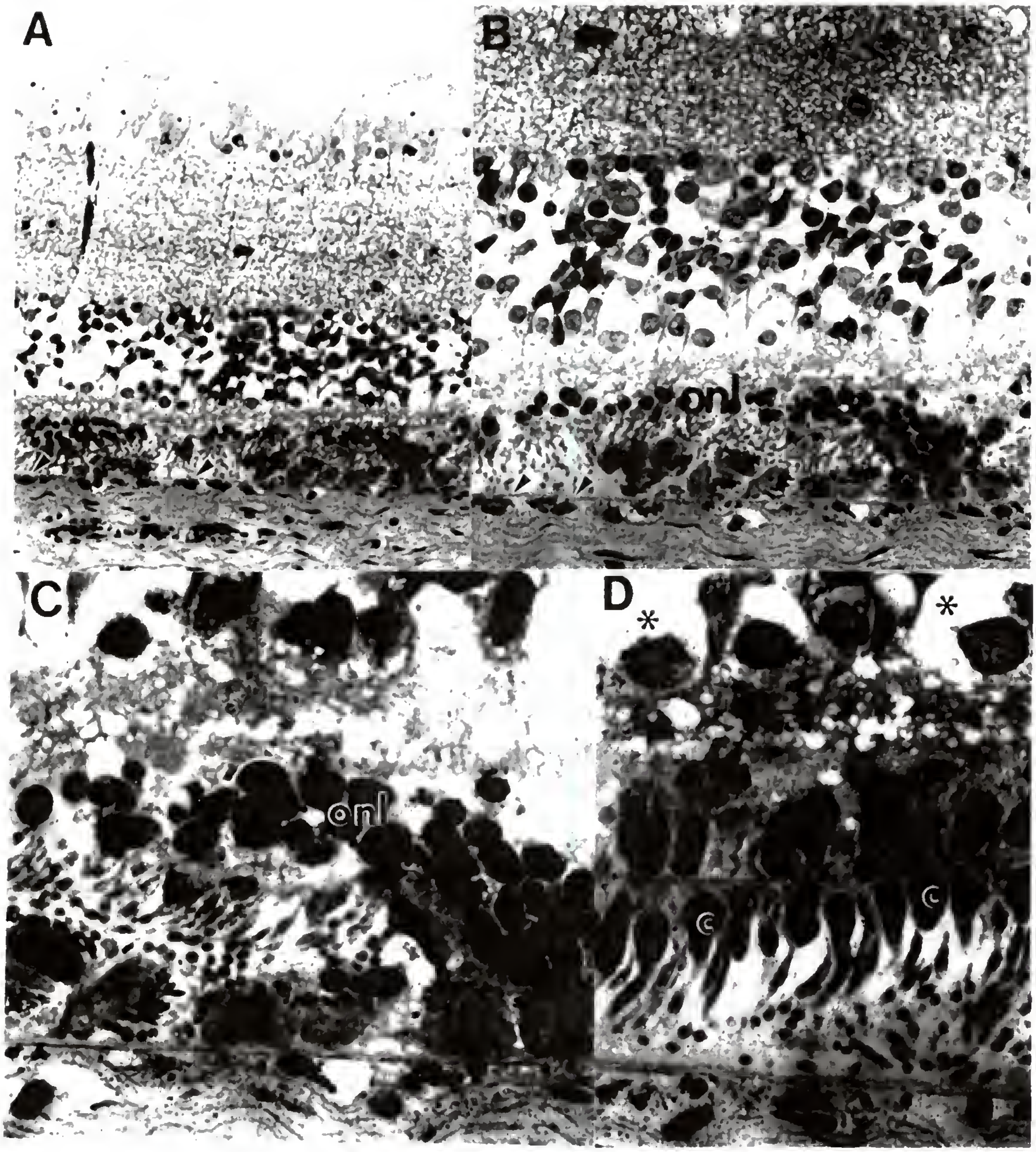
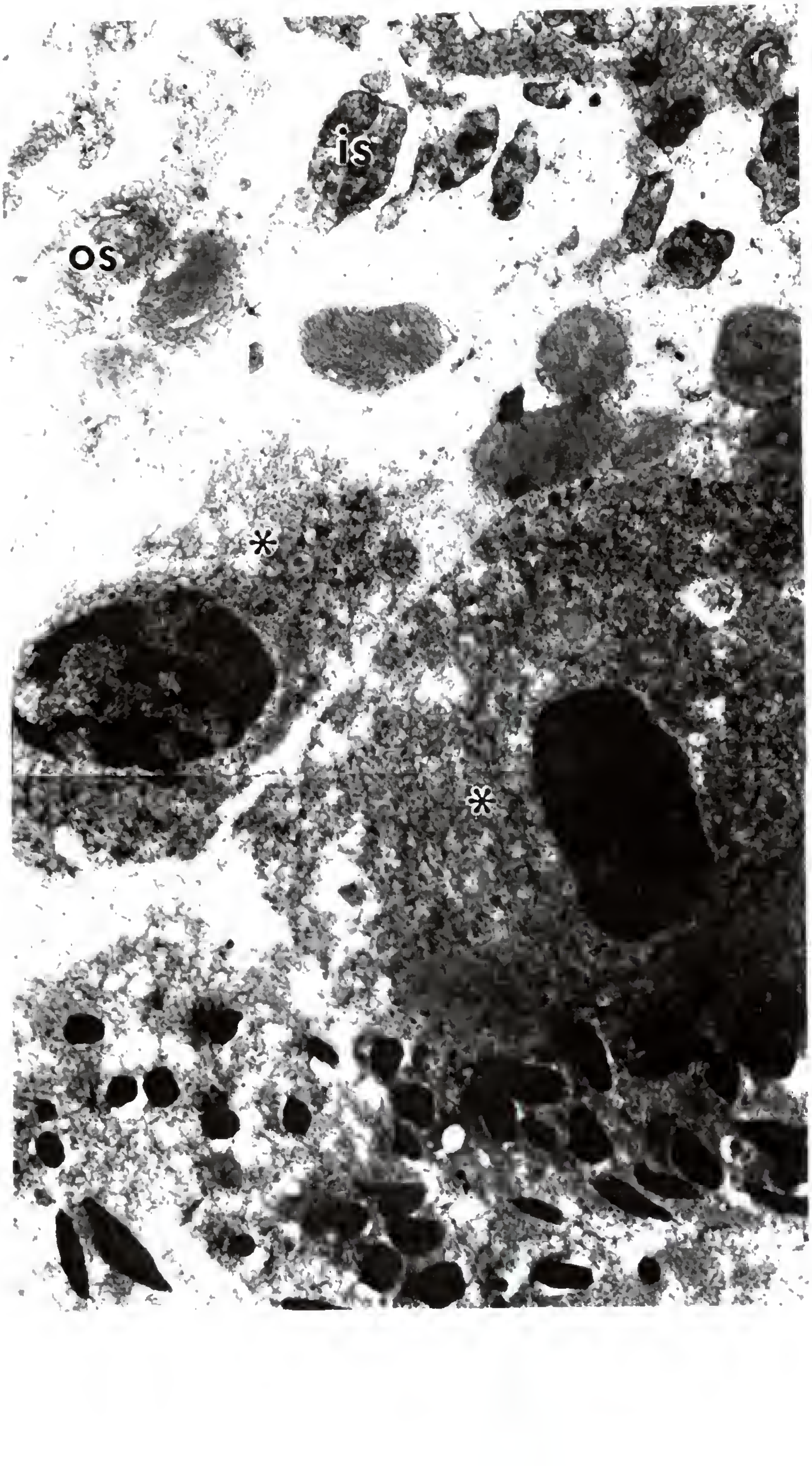

A
B
onl
C
onl
D
*
*
c
c

Figure 43: Animal fed the Zn+ diet for 11 days, exposed to 72 hours of continuous light and sacrificed 3 days post-exposure. Severe retinal damage. The severe degenerative changes in make it difficult to identify the cell types. Most OS and IS are degenerative. Detaching RPE cells (asterisks)) are within the OS layer and degenerative, and degenerative ONL cells are present. The migrating RPE have lost many of their structural and functional specializations i.e. they lack melanin and microvilli. The ELM is not distinguishable.(10,000x)


is
os
*
*

pattern and in many cases pyknosis. INL cells were also degenerative and in many cases were pyknotic. (Figure 44). The peripheral retina of this eye was less damaged than the central retina, but exhibited similar changes. The other eye of this animal had moderate damage with multifocal RPE pyknosis, alteration of most ONL nuclei with densification of chromatin and a few pyknotic cells and some areas of outer and inner segment disruption. The central retina was more damaged than the peripheral retina in this eye. The inner retinal layers appeared edematous with a few dying cells in the INL (Figure 45).

Compared to the animals fed the zinc deficient and normal diets, the melanin granules appeared dense and exhibited infrequent translucency or fragmentation despite significant RPE damage. However, in one animal with mild focal damage, melanin in the RPE and choroid also exhibited these features. In some choroidal melanocytes, occasional melanolysosomes were observed (Figure 46).

A summary of the damage found in the central and peripheral sections of both eyes of each animal is shown in Table 10. For this table if an eye exhibited only mild pathologic changes it was included in the "normal" category. Only if significant damage was present was a section designated as having damage. If one compares between groups, 41.67% of the sections analyzed in the animals on the zinc normal diet had damage, 25% of those on the zinc deficient diet and 41.67% of those on the zinc supplemented diet. None (0%) of the eyes of the control animals that were not exposed to light stress had significant damage. However, the control animal on the zinc deficient diet for 10 days had mild degeneration of the retina with disorganization of the OS and IS as well as some ONL cell death.

Figure 44: Animal fed the Zn+ diet for 11 days, exposed to 72 hours of continuous light and sacrificed 3 days post-exposure. Severe retinal damage.

a) Pyknotic rod (r) nuclei and chromatolysis of cone (c) nuclei in the ONL. (10,000)

b) Most INL nuclei were pyknotic and many exhibited cytoplasmic edema (asterisk). (2000x).

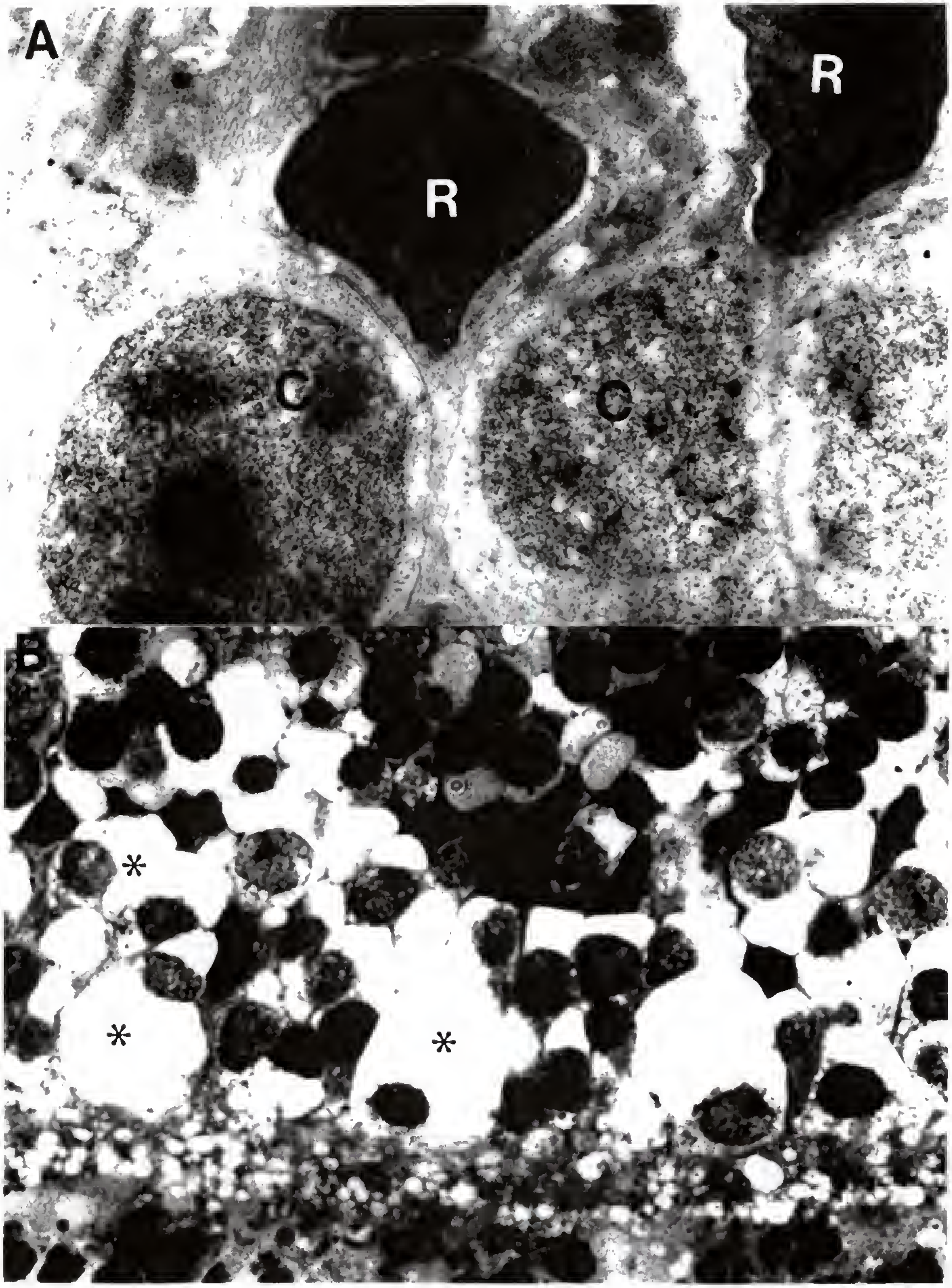

A
R
R
C
C
B
*
*
*

Figure 45: Animal fed the Zn+ diet for 11 days, exposed to 72 hours of continuous light and sacrificed 3 days post-exposure. Damage was less severe in the opposite eye of the animal shown in Figures 42-44.

a) Peripheral retina has a few degenerative ONL and RPE cells (arrowheads). (400x) b) The central retina was more severely damaged with edema in all layers and densification of all rod nuclei and some cone nuclei. Cones appear more resistant to the light damage as they exhibit edema but less frequently pyknosis. RPE cells are edematous and one is undergoing pyknosis (arrow) A few INL cells were degenerative (arrowheads). (800x)

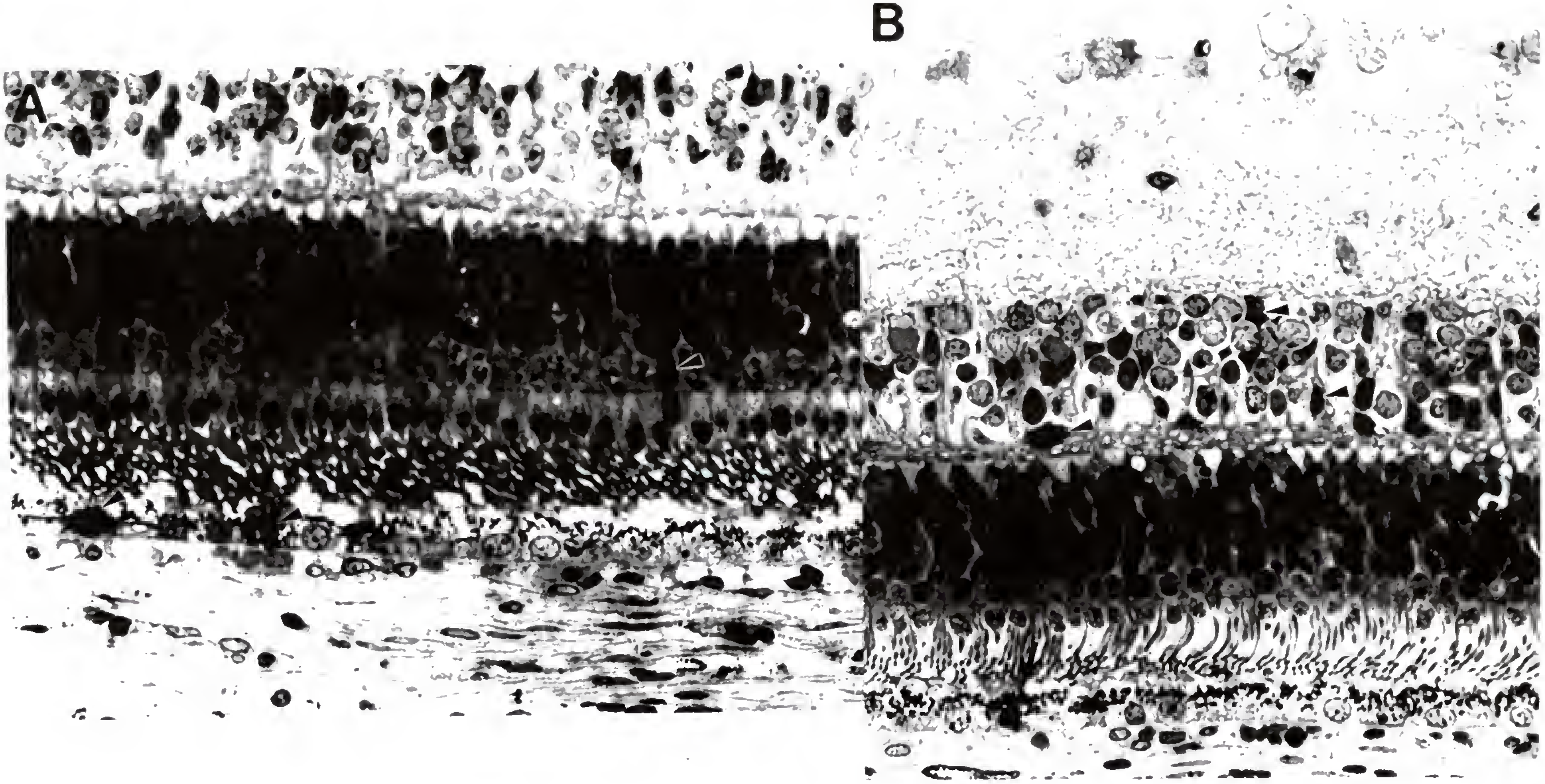
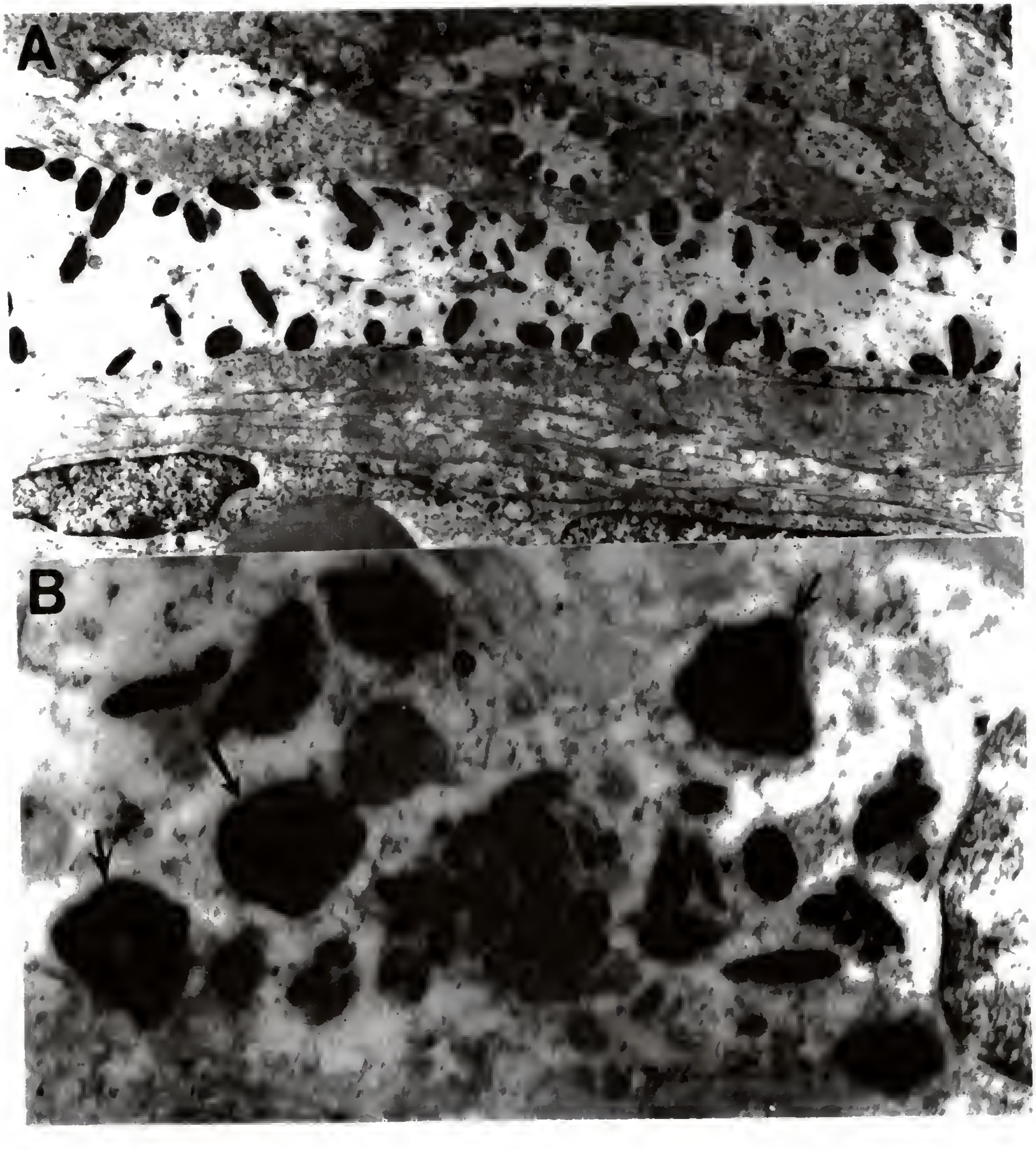

A
B

Figure 46: Animal fed the Zn+ diet for 11 days, exposed to 72 hours of continuous light and sacrificed 3 days post-exposure.

a) Choroidal melanocyte with numerous fragmenting melanin granules and intracellular edema.(10,000x) b) Choroidal melanocyte with melanolysosomes (arrows) suggesting melanin degradation is occurring. (20,000x)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Since three animals were in each group in Part II of the experiment, quantitative assessment of photic injury was done utilizing measurement of ONL nuclear density and % dying nuclei. Retinas from control animals, i.e., those on the experimental diets but did not receive light stress, were also counted (Table 11). Results of counts on retinas of animals sacrificed 72 hours post-exposure are presented in Table 12. There was no difference in the outer nuclear layer density between exposed and unexposed groups regardless of dietary zinc level ($p > 0.05$). The mean percent dying cells in the three diet groups for control animals were Zn- 1.02 +/- .39%, ZnN 0.69 +/- .69% and Zn+ 0.64 +/- .18%. For animals exposed to 72 hours of continuous light stress the mean percent cell dying for the diet groups were Zn- 24.56% +/- 37.18, ZnN 14.9 +/- 15.87%, and Zn+ 27.14 +/- 39.29%. The small number of control animals and the wide variability in degree of damage in each diet group decreases the power of the analysis, and the ANOVA indicated that the percent cells dead significantly differed between continuously exposed and unexposed eyes in the high zinc diet group only ($p = 0.034$). When the kidney MT level was used as a zinc status covariate however, and the arcsine-square root form of percent dying cells was used there was a significant difference between the unexposed and continuously exposed animals in all diet groups ($p = 0.048$). Although not significant there was trend for animals with lower kidney MT values to exhibit increased percent cells dying in both the control and light exposed animals (Figure 47).

Table 11: Control animal outer nuclear layer cell density and percent dying. Five areas of each section were counted. Total number of nuclei in a defined area was determined and then the dying nuclei counted to determine the percent dying. Compared to Table 6 there is a significantly less % dying nuclei. The ONL cell density does not appear to differ from light treated animals.

Animal/ eye	Diet	ONL/mm^2	% dying
181od	ZnN	30600	1.66
181os	ZnN	21200	0.54
7180od	ZnN	22860	0
7180os	ZnN	25300	0.56
125os	Zn-	20000	1.3
125od	Zn-	21900	0.75
126os	Zn+	21600	0.77
126od	Zn+	25400	0.52

Table 12: ONL nuclear counts and percent dying in animals exposed to 72 hours of continuous light and sacrificed 3 days post-exposure. Note the significant increase in % dying nuclei in the animals exposed to light compared to controls in Table 11. ONL cell density is not different from control eyes.

Animal / eye	Diet	ONL/mm^2	% dying
178od	ZnN	24,200	23.00
178os	ZnN	21,200	24.98
363os	ZnN	24,700	0.29
363od	ZnN	23,500	2.70
180os	ZnN	24,800	0.68
180od	ZnN	28,400	37.88
179os	Zn-	26,900	10.40
179od	Zn-	23,300	22.00
182od	Zn-	34,300	0.54
182os	Zn-	23,600	16.05
364od	Zn-	23,700	98.40
364os	Zn-	22,900	0.00
365os	Zn+	22,400	6.04
365od	Zn+	27,500	0.83
176od	Zn+	29,300	1.54
176os	Zn+	23,000	6.30
177os	Zn+	22,800	97.40
177od	Zn+	27,200	50.70

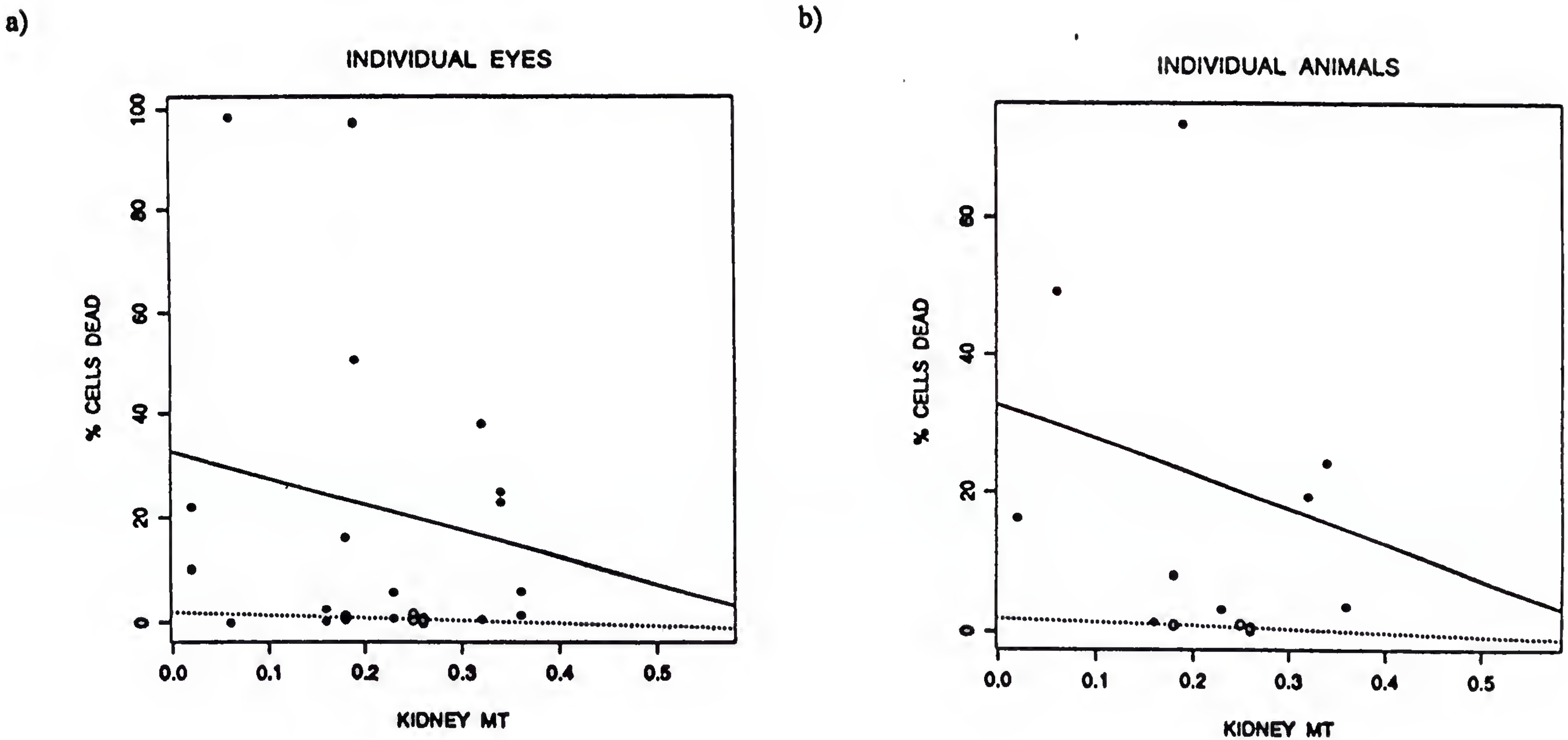


Figure 47: Relationship between kidney metallothionein and degree of damage. The percent of cells dying is compared to kidney metallothionein level in each animal. Whether the eyes (a) or animals (b) are used it apparent from these figures that lower kidney MT levels are associated with higher percent dying nuclei. It is notable that this trend occurred in both control (open circles) and light-exposed (dots) animals / eyes. The finding, however, was not statistically significant and only indicates a trend.

Part 2: Continuous Light Stress (72h); 3 weeks Post-Exposure (31 days on diet)

Table 13 presents the results of counts of percent dying and ONL density on the three animals (each on a different diet) that were sacrificed 3 weeks after exposure and of the three animals that served as controls and did not receive light stress. Details of morphologic changes are discussed below. Melanogenesis was observed in neither of the eyes of the animal on the ZnN diet, in one eye of the animal on the Zn+ diet and in both eyes of the animal on the Zn- diet.

Zinc normal diet (n=1). The retina of one eye was normal. In the other eye, a focus of degeneration with few pyknotic and displacing ONL nuclei was present in an otherwise normal retina. The RPE, outer and inner segments were normal. Some ONL nuclei that had displaced through the ELM had normal chromatin appearance (Figure 48). This was considered evidence of recovery from previous damage.

Zinc deficient diet (n=1). Both eyes of this animal appeared to have very mild degeneration. The central retina in one eye had a foci of mild degeneration with a few pyknotic ONL nuclei (arrowheads) (Figure 49). The peripheral retina had a few normal appearing photoreceptor nuclei displaced through the ELM. Normal appearing IS were present in an abnormal position on the surface of the RPE. Both of these observations were considered evidence of retinal recovery from the previous light stress. The outer segments were mostly normal on light and electron microscopic examination except for poor insertion into the RPE microvilli and occasional disorganization. The inner retinal layers were normal.

Table 13: ONL counts and % dying in light treated and normal eyes three weeks post-exposure to 72 hours of continuous light. There was no significant difference in ONL density or % dying cells between light treated and control eyes. The light treated eyes had a slight increase in the percent dying nuclei but this is not statistically significant since only one animal was analyzed for each experimental diet.

Light stress 3 weeks post-exposure			
Animal and eye	Diet	ONL/mm2	% dye
361od	ZnN	26800.00	2.48
361os	ZnN	31600.00	0.00
362os	Zn-	22700.00	5.20
362od	Zn-	23500.00	0.00
366od	Zn+	25600.00	1.80
366os	Zn+	24400.00	0.17
Control 3 weeks post-exposure			
110os	ZnN	24800.00	0.00
110od	ZnN	30600.00	0.15
111od	Zn-	23700.00	0.00
111os	Zn-	27600.00	0.30
109os	Zn+	28000.00	1.30
109od	Zn+	25900.00	0.00

Figure 48: Animal fed the ZnN diet, exposed to 72 hours of continuous light and sacrificed 3 weeks post-exposure. No significant damage or cell loss was present. Displaced normal appearing nuclei were observed. This retina must have recovered from any damage that occurred as a result of light exposure.

a) Normal retina other than two displaced cone nuclei in the IS layer (arrows). (800x)

b) Higher magnification confirms the normal ultrastructure of the displaced cells as well as all the outer retinal layers.(2000x)

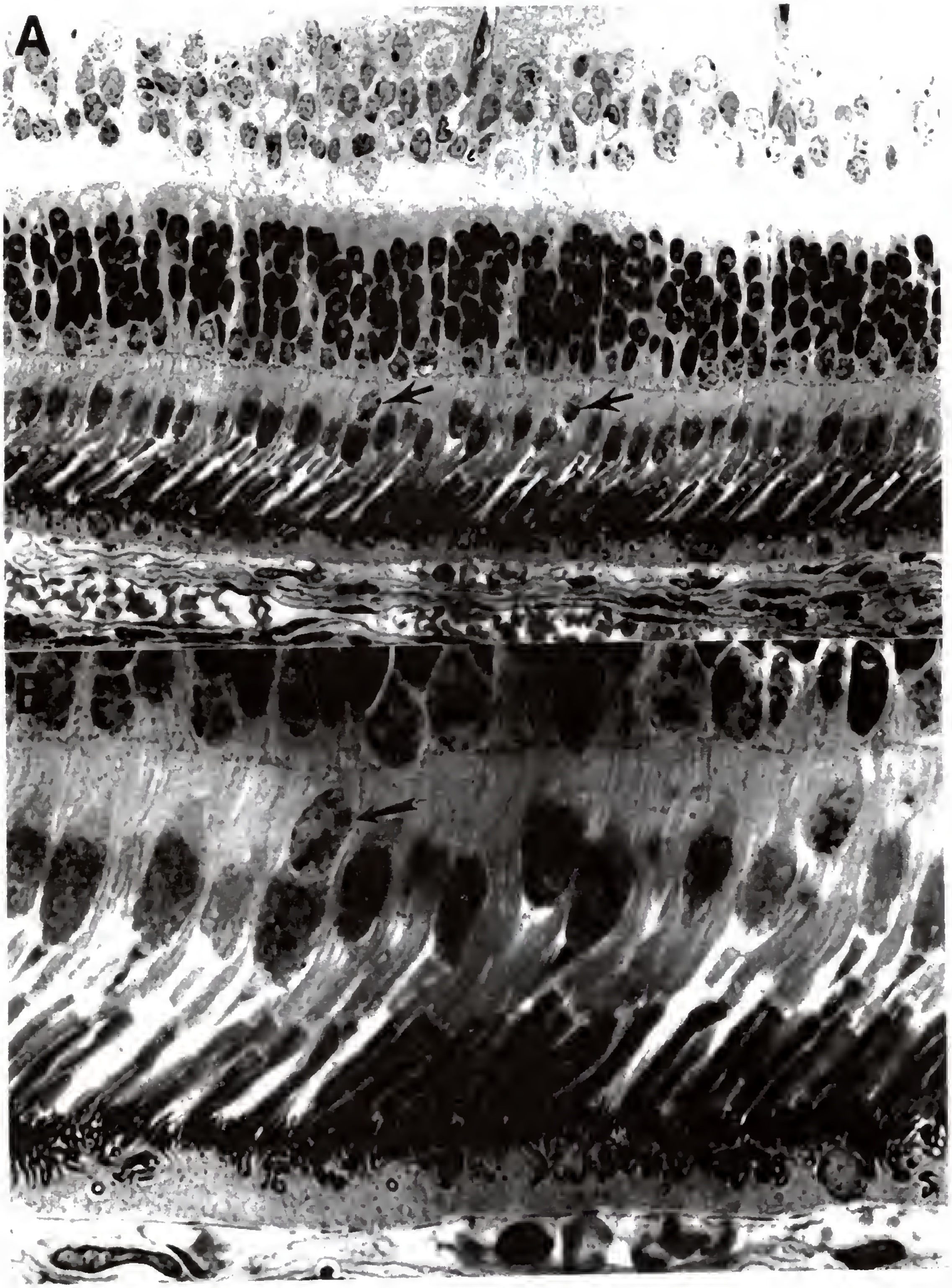
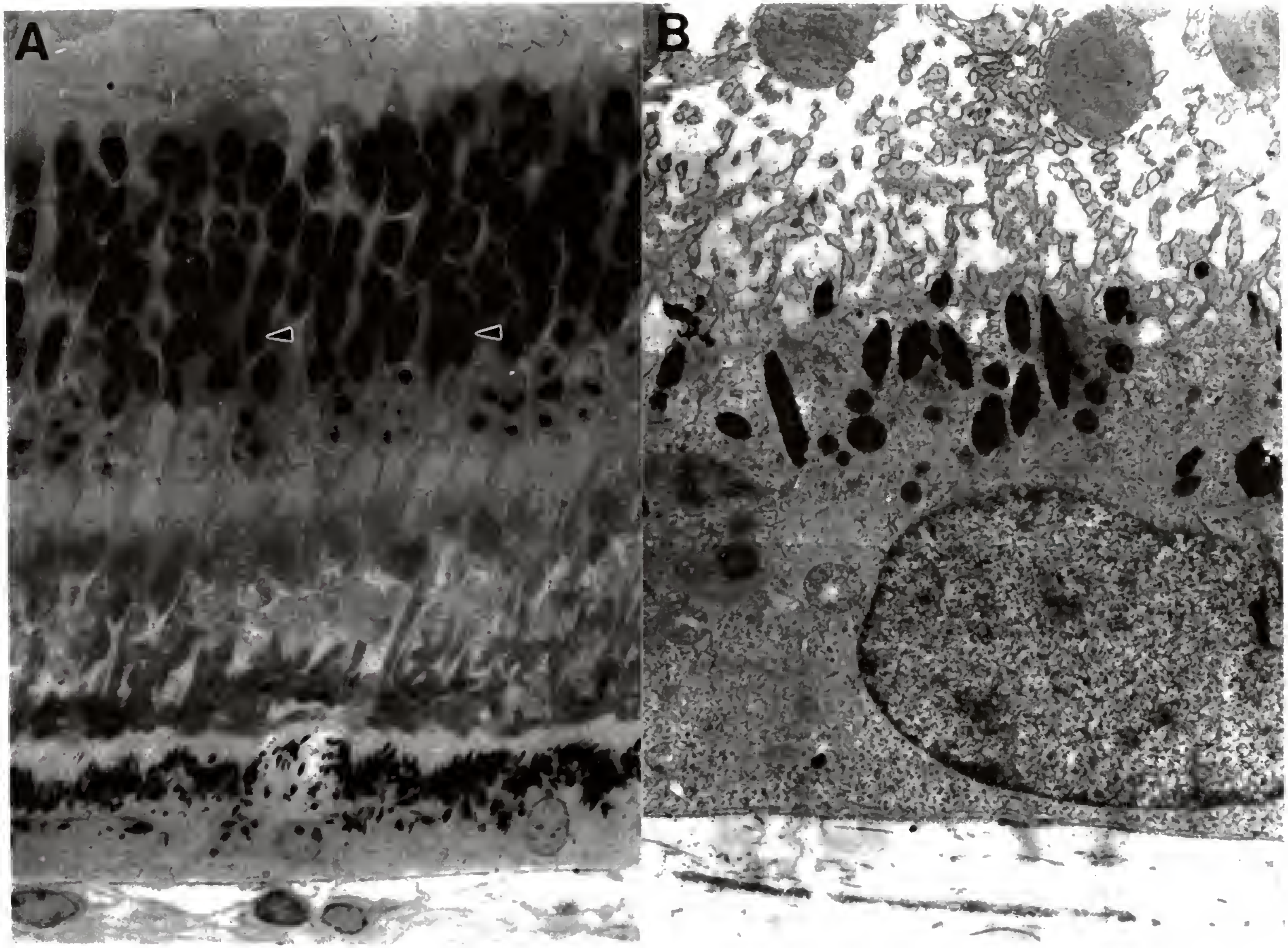

A
B

Figure 49: Animal fed the Zn- diet, exposed to 72 hours of continuous light and sacrificed 3 weeks post-exposure. The retinas showed mild degenerative changes.

a) A few ONL exhibit chromatin densification (arrowheads). The OS layers is slightly disorganized and poorly inserted into the microvilli of the apical RPE. (2000x) b) Normal RPE with weak attachment between the RPE and OS. (10,000x)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Zinc supplemented diet (n=1). The retinas of both eyes were normal except for the occasional displaced IS within the outer segment layers. Ultrastructurally these IS appeared normal. Again this was considered evidence of recovery. In contrast to the zinc deficient animal the outer segments are firmly embedded in the RPE microvilli (Figure 50). The RPE melanin granules in many cases had small areas of translucency that may indicate prior bleaching from light exposure. In some areas mild disorganization of OS was present as well as some membranous debris on the surface of the RPE (Figure 51).

Control lighting,:14 days on diet. Due to restraints of cost for housing and feeding the pigs the controls in part 1 of the study were utilized for part 2 of the study as well. One animal on a casein-sucrose based normal zinc diet served as an additional control for part 2 of the experiment. This animal received the casein-sucrose diet for 14 days while Part 2 of the study was being performed. The retinas of this animal were normal except for a few degenerating nuclei in the outer nuclear layer (Figure 8).

Immunohistochemistry

One micron sections of liver, kidney, and ocular tissues were stained using a gold labeled antibody technique enhanced by silver intensification. Ultrathin sections were placed on copper grids and also stained with the immunogold technique. The primary antibody was the rabbit anti-porcine MT IgG. The secondary antibody was a gold-labeled goat anti-rabbit IgG antibody. Consistent results could not be generated at the light or electron microscopic level. Several dilutions were evaluated, i.e., 1:4, 1:10, 1:100, 1:400, 1:1000 but only low dilutions such as 1:4 and 1:10 yielded staining. Background staining was high in

Figure 50: Animal fed the Zn+ diet, exposed to 72 hours of continuous light and sacrificed 3 weeks post-exposure. No significant degeneration was observed in either eye.

a) Some displaced but normal appearing nuclei are in the IS layers and some IS were found on the surface of the RPE (arrowhead). (2000x) b) RPE-OS interdigitations were normal in these eyes vs. those of animals fed the Zn- diet for three weeks (Figure 49). Some of the melanin granules have small areas of translucency. (20,000x) c) Normal mitochondria in IS on the surface of the RPE. (20,000x)

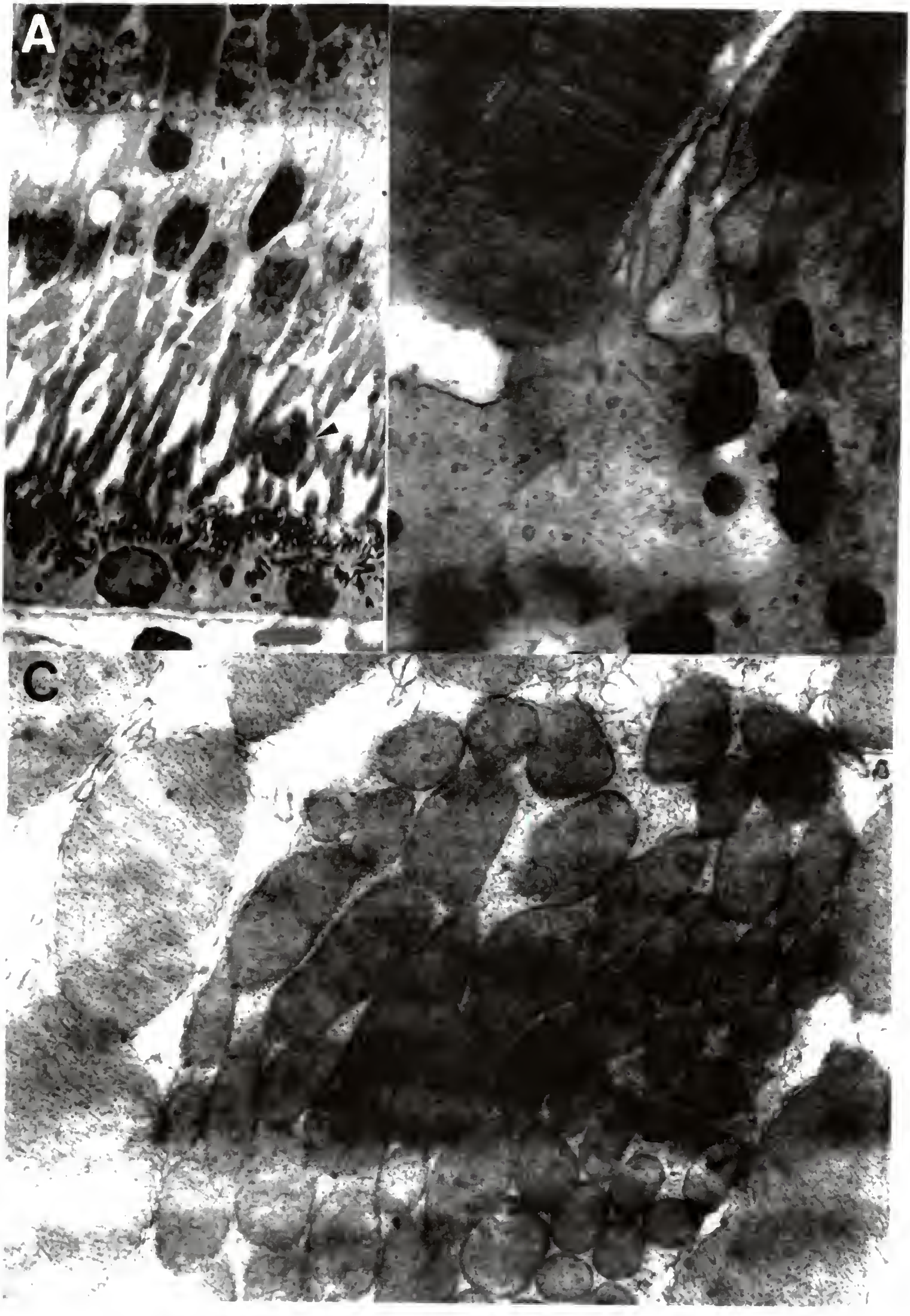
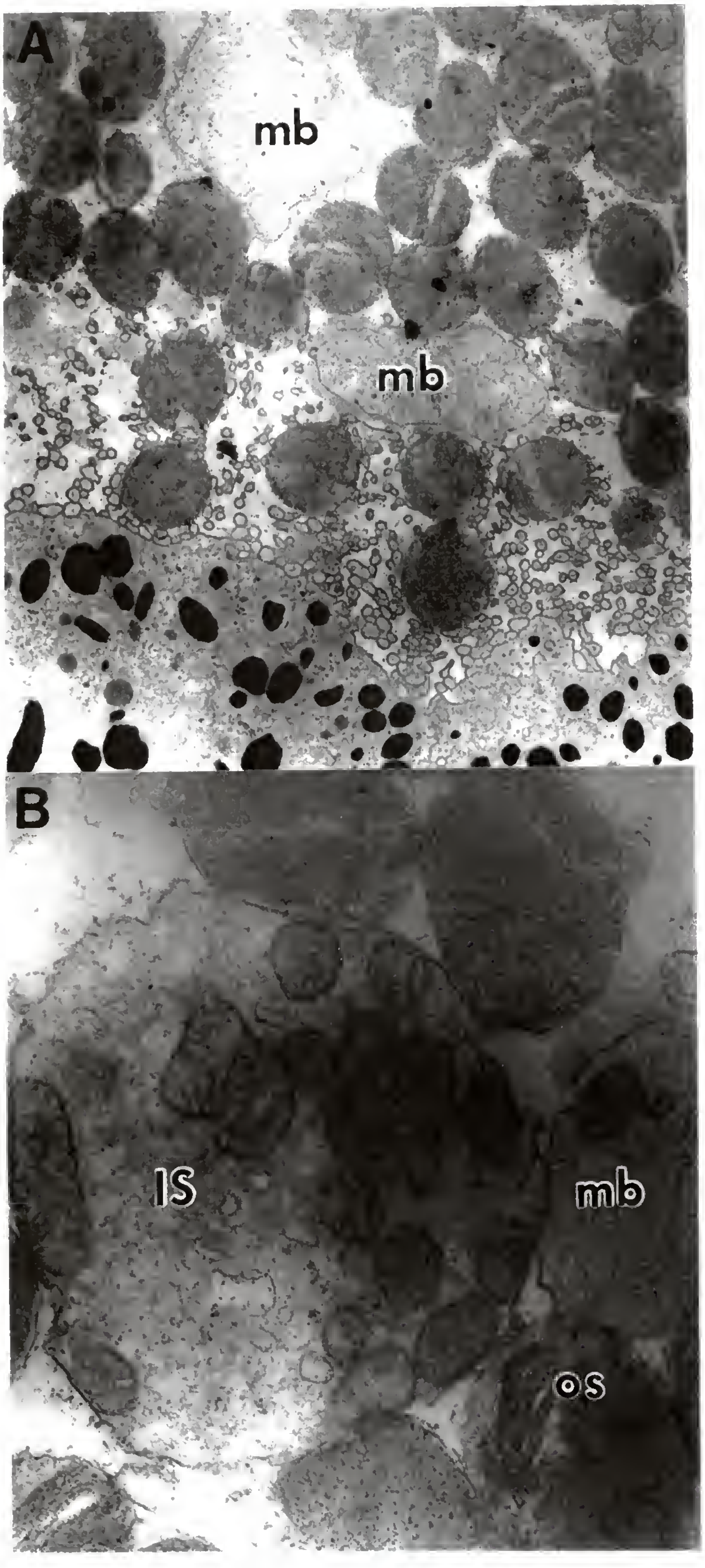

A
C

Figure 51: Animal fed the Zn+ diet, exposed to 72 hours of continuous light and sacrificed 3 weeks post-exposure. Some areas had not completely recovered from damage as OS and IS debris were still present in the OS layer and some OS disorganization was observed.

a) RPE-OS interface with membranous debris (mb) without any internal structure may be OS or IS debris. Note the normal melanin granules. (10,000x) b) Higher magnification of IS debris among OS with an adjacent disorganized OS. (24,000x)


A
mb
mb
B
IS
mb
os

all trials which precluded accurate localization of MT in tissues. A peroxidase-anitperoxidase staining kit was also tested on liver and ocular tissues. A similar negative result with various trials suggested the antibody was not in high enough titer to accurately detect MT in tissues. Because of these difficulties the results of immunohistochemistry staining are not presented.

Metallothionein Assay

Results of the cadmium-hemoglobin binding assay on liver and kidney tissues are shown in Tables 5-8 and 14. Due to the small amount of retinal and choroidal tissue available from the experimental animals, retinal and choroidal tissues from normal pigs at a slaughter house were obtained to test the assay. The sensitivity of the assay using the 2.0 μg/ml $CdCl_2$ solution with 0.5 μCi/ml of Cd^{109} was not sufficient to detect MT in retinal and choroidal tissues. To increase the sensitivity of the assay, a 1 μg/ml CdCl solution with 1 μCi of Cd^{109} was utilized. This type of modification, i.e., increasing the amount of radioactive cadmium, was suggested by Eaton and Toal [181] and was performed on RPE cell cultures by other investigators.[12] With this modification the assay could be performed on the retina but still would not work on the choroidal-RPE tissue due to the small amount of tissue available and because the tissue was difficult to homogenize without loss of sample. The kidney, liver and retinal MT values are shown in Tables 14-16. Results of copper and zinc determinations by atomic absorption spectrophotometry are also given in Tables 14-16.

Recall that in order for the assay to work on ocular tissue the neural retinas from both eyes of an animal were combined and analyzed by the Cd-Hb binding assay. The mean eye MT for the different diet groups are shown in Tables 14-16. For control animals the eye MT

Table 14: Kidney (K), liver (L) and retinal metallothionein (Eye MT), copper (Cu) and zinc (Zn) values from tissues of control animals. These animals were not exposed to high intensity light and were fed one of three experimental diets that contained low (Zn-), normal (ZnN) or high (Zn+) levels of zinc. The cadmium-hemoglobin binding assay was utilized to determine the level of MT. Tissue Zn and Cu levels were determined by atomic absorption spectrophotometry. Protein (PR) measurement for each sample allowed expression of MT as µg MT/mg protein which enables more valid comparison between tissues that have different water content. The number of days each diet was fed is also shown.

Animal	Diet	days on diet	Eye MT µg/mgPR	K Zn µg/g	K MT µg/mgPR	L Zn µg/g	L MT µg/mgPR	L Cu µg/g	K Cu µg/g
181	ZnN	13	0.05	37	0.25	47	0.76	15.9	5.39
7180	ZnN	13	0.07	29.8	0.26	56	1.7	3.9	7.14
110	ZnN	29	0.01	30.3	0.54	44	0.57	3.3	4.67
125	Zn-	12	0.02	28.4	0.18	45	0.76	3.55	2.87
111	Zn-	29	0.01	32.2	0.18	51	0.23	2.7	2.96
126	Zn+	11	0.02	35.2	0.26	45	0.54	3.55	4.41
109	Zn+	29	0.01	45.3	0.61	57	1.39	4.32	6.29

Table 15: Kidney (K), liver (L) and retinal metallothionein (Eye MT), copper (Cu) and zinc (Zn) values from animals exposed to 36 (36hl) or 72 (72hI) hours of high intensity intermittent light and fed the different experimental diets (Zn-, ZnN, and Zn+). The number of days each diet was fed is also shown.

Animal	Diet	Light	days on diet	Eye MT µg/mgPR	K Zn µg/g	K MT µg/mgPR	L Zn µg/g	L MT µg/mgPR	L Cu µg/g	K Cu µg/g
121	Zn-	36hI	13	0.04	32.2	0.2	48	0.52	5.95	3.73
114	ZnN	36hI	16	0.03	37.8	0.22	37	0.47	3.55	6.03
115	Zn+	36hI	12	0.02	26.7	0.28	67	2.14	3.21	3.9
119	Zn-	36hI	30	0.06	30.7	0.35	64	2.53	4.58	4.75
112	ZnN	36hI	31	0.03	31	0.23	43	0.3	3.3	4.58
124	Zn+	36hI	29	0.04	41	0.4	122	6.14	5.78	7.06
123	Zn-	72hI	15	0.03	32.3	0.27	54	1.26	4.41	3.55
117	ZnN	72hI	13	0.05	37.8	0.3	60	2.13	3.3	5.69
116	Zn+	72hI	14	0.03	25.3	0.42	62	1.55	3.93	2.87
120	Zn-	72hI	32	0.02	26.3	0.17	28	0.16	3.04	2.44
118	ZnN	72hI	31	0.06	28.7	0.45	46	1.12	2.7	8
122	Zn+	72hI	32	0.06	33.2	0.33	68	1.51	5.26	5.69

Table 16: Kidney (K), liver (L) and retinal metallothionein (Eye MT), copper (Cu) and zinc (Zn) values from animals exposed to 72 hours of continuous high intensity light and fed the different experimental diets (Zn-, ZnN, and Zn+). The number of days each diet was fed is also shown.

Animal	Diet	Light	days on diet	Eye MT µg/mgPR	K Zn µg/g	K MT µg/mgPR	L Zn µg/g	L MT µg/mgPR	L Cu µg/g	K Cu µg/g
178	ZnN	72hC	13	0.03	35.1	0.34	66	1.36	43.8	11.61
363	ZnN	72hC	12	0.08	36.8	0.16	95	1.12	242	7.55
180	ZnN	72hC	13	0.03	33.6	0.32	69	2.74	11.1	4.85
179	Zn-	72hC	13	0.05	30.7	0.02	64	0.26	17	13.77
182	Zn-	72hC	13	0.05	26.1	0.18	36	0.36	9.17	3.23
364	Zn-	72hC	13	0.04	38.5	0.06	81	1.05	10.8	6.2
365	Zn+	72hC	13	0.07	31	0.23	66	1.07	39.7	3.77
176	Zn+	72hC	13	0.02	31.2	0.36	62	1.64	13	6.2
177	Zn+	72hC	13	0.04	32.2	0.19	65	1.81	14.9	5.12
361	ZnN	72hC	31	0.06	32.9	0.53	84	4.53	14.9	10.8
362	Zn-	72hC	31	0.09	32.9	0.13	47	1.1	161	4.31
366	Zn+	72hC	31	0.09	39.5	0.78	127	6.27	18.4	9.44

levels for each diet group were Zn- .02 (n=1), ZnN 0.06 +/- .01 (n=4), and Zn+ .02 +/- 0 (n=2) µg MT/ mg protein. For the intermittent light group the 36 hour and 72 hour exposure groups were combined so that 2 animals were in each diet group. The mean eye MT levels for the intermittent light-exposed diet groups were Zn- .03 +/- .01, ZnN .04 +/- .01, and Zn+ .06 +/- .03 µg MT/ mg protein. The mean eye MT levels for the continuous light-exposed diet groups (n=3 in each group) were Zn- .04 +/- .01, ZnN .05 +/- .02, and Zn+ .04 +/- .02 µg MT/ mg protein. There was no significant effect of dietary zinc on the eye MT level regardless of light-exposure group. If diet is not considered and the eye MT level of each exposure group are compared, i.e., unexposed, intermittent and continuous, there is a trend toward increased eye MT levels in animals exposed to light vs. those not exposed, but this was not statistically significant probably due to the small number of control animal eye MT values. Eye MT was not significantly correlated with kidney MT levels nor with the degree of damage or percent dying nuclei.

Several Pearson correlation analyses were performed. The liver zinc level correlated well with liver metallothionein ($r = 0.8$). Kidney zinc levels were not well correlated with kidney metallothionein ($r = 0.32$). Kidney zinc and liver zinc levels were not significantly correlated with an $r = 0.51$. Kidney and liver metallothionein levels were not significantly correlated with an $r = 0.53$. Copper levels in the liver or kidney did not correlate with metallothionein levels in the same organs for liver $r = -0.05$ or kidney $r = 0.2$. Ocular metallothionein levels did not correlate with liver or kidney zinc levels.

Energy Dispersive X-ray Microanalysis of RPE and Choroidal Melanin Granules

Elemental analysis was performed on control animals not exposed to light and on animals from Part 2 of the experiment (72 hours of continuous light stress). As mentioned in the methods the peak-to-background (PK/BG) ratios were determined by measuring the elemental peak of interest, i.e., calcium and the height of the adjacent background of the spectra at the base of the peak (Figure 2). Therefore, when interpreting PK/BG ratios a value of 1.0 indicates no significant peak is present above background, i.e., that element is not present. Table 17 summarizes the mean PK/BG ratios for Ca, Fe, Cu and Zn in unexposed and exposed animals separated by diet. Examination of the table reveals several trends but none were statistically significant.

Within an animal there was often a marked difference in elemental content between eyes in both the control and light stressed groups (Figure 52). In some eyes however there was close agreement in elemental content of the melanin granules (Figure 53). In almost all eyes examined, choroidal melanocyte granule PK/BG ratios of Ca were higher than those in the RPE although this was not significant. In cases where zinc and calcium PK/BG ratios were high, the calcium and zinc peaks had a tendency to vary inversely (Figure 54). In most cases there was little variation in the spectra of individual granules within an eye but there were eyes in which there was a greater degree of variation between melanin granules as the standard deviation of mean PK/BG ratios was large (Table 17).

Table 17: Mean PK/BG ratios of Ca, Fe, Cu and Zn melanin granules in the RPE and choroid in the eyes of control and light-exposed animals on the different experimental diets. Std = standard deviation.

	RPE MELANIN						CHOROID MELANIN					
Diet	Zn-		ZnN		Zn+		Zn-		ZnN		Zn+	
	PK/BG	Std	PK/BG	Std	PK/BG	Std	PK/BG	Std	PK/BG	Std	PK/BG	Std
Calcium												
C	2.23	.79	3.97	1.73	2.15	.04	2.36	.71	5.91	2.36	4.61	.95
72hC	4.01	1.47	4.28	1.34	3.01	1.58	7.23	3.69	6.19	2.22	4.43	1.45
Iron												
C	1.39	.18	1.22	.17	1.37	.09	1.09	.02	1.02	.03	1.07	.09
72hC	1.47	.28	1.19	.17	1.26	.17	1.20	.26	1.08	.13	1.06	.06
Cu												
C	1.09	.12	1.39	.26	1.25	.04	1.00	.01	1.40	.25	1.46	.30
72hC	1.47	.29	1.48	.34	1.43	.26	1.39	.42	1.43	.57	1.41	.31
Zn												
C	1.22	.30	1.66	.27	1.39	.33	1.27	.15	1.48	.26	1.49	.38
72hC	1.76	.29	2.04	.23	1.84	.73	1.22	.24	2.28	1.69	2.40	1.17

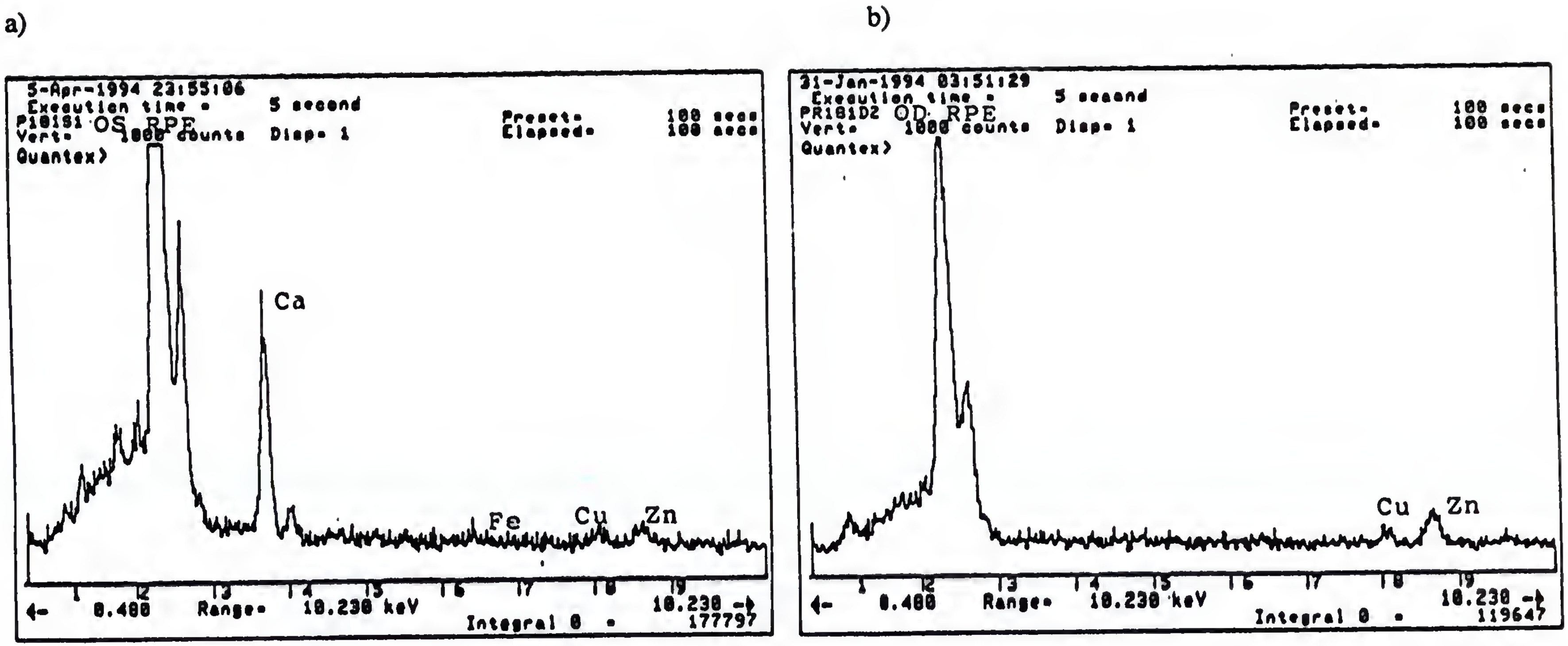


Figure 52: Variation of elemental spectra between eyes. Elemental spectra from RPE melanin granules of both eyes of an animal that was fed the normal zinc diet and was not exposed to light.

a) Typical spectra from the RPE of the left eye had a high peak of Ca, and smaller peaks of Fe, Cu and Zn. b) A spectra obtained from the right eye lacked the high Ca peak has a similar Cu peak and a slightly higher Zn peak. An iron peak is present but is barely above background levels.

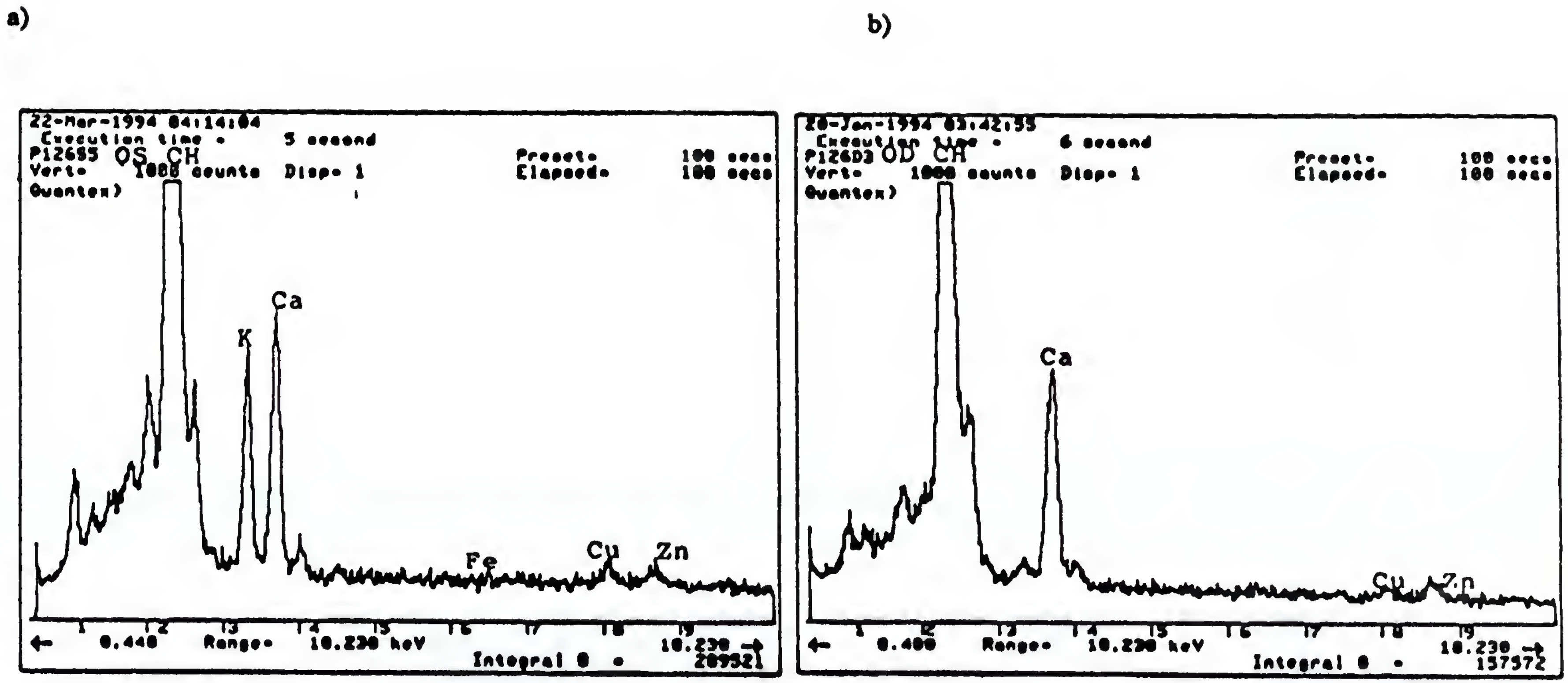


Figure 53: Similarity of elemental spectra between eyes. Control animal fed the Zn+ diet for 12 days. This animal has very similar elemental spectra when comparing both eyes. It is apparent that feeding the diet for this short amount of time did not significantly influence the zinc peak in choroidal melanin granules. Note the lack of an iron peak in choroidal melanin granules compared to the RPE spectra in Figure 52.

a) Left eye has Ca, Cu and Zn peaks but also has a relatively high potassium (K) peak. Potassium often is lost during processing due to its solubility but with the slam freezing technique specimens often have a K peak. b) Right eye.

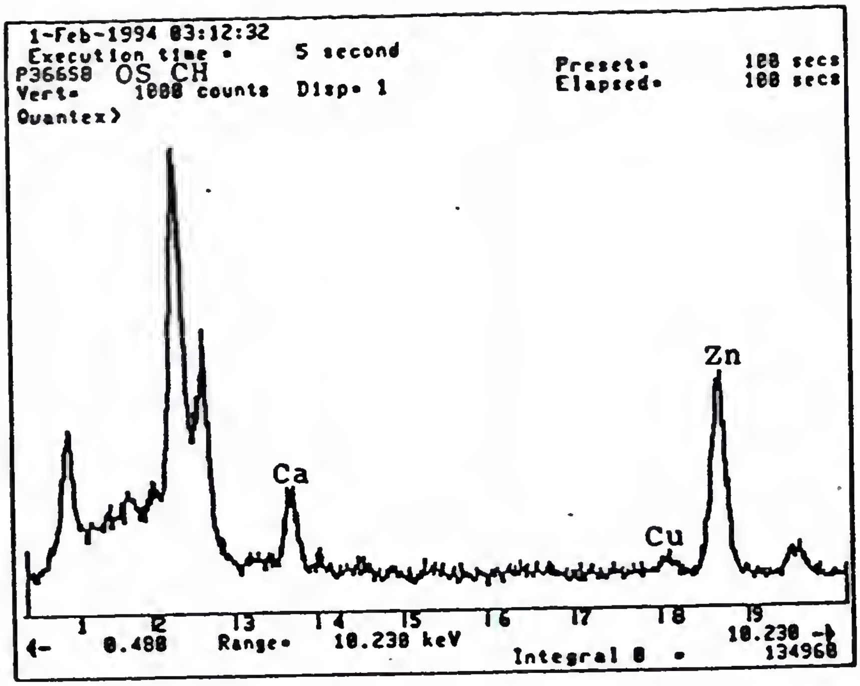


Figure 54: Effect of diet on elemental spectra. This animal was exposed to 72 hours of continous light and fed the Zn+ diet for 31 days. The biochemical data suggested a positive zinc balance and in this case the melanin granules of the eye appeared to be affected by dietary zinc. A typical spectra from a chorodial melanin granule is shown. The inverse relationship between the Zn and Ca spectra is demonstrated here as the Ca PK/BG ratio is lower than usual for choroidal melanin granules and the Zn peak is markedly higher. The Zn level was so elevated in this granule that in addition to the primary $K_{\alpha 1}$ line at 8.638 KeV, the $K_{\beta 1}$ (Kb) at 9.572 KeV and $L_{\alpha 1}$ (L) at 1.011 KeV lines are also present.

In all animals the elemental content of the choroid was highly correlated with that of the RPE. For all animals regardless of light exposure, the correlation coefficients for RPE and choroidal Ca, Fe, Cu and Zn were respectively 0.74, 0.52, 0.86 and 0.84 all with $p = 0.0001$. It is apparent that the level of iron in the RPE and choroid was not as highly correlated as the other elements. Examination of the Fe in the spectra revealed that the choroidal melanin had much less iron bound compared to the RPE spectra. For the mean Zn PK/BG ratios in choroid and RPE, correlation coefficient values were: $r = 0.9$ for all animals, 0.958 for control animals and 0.845 for those exposed to light (for all $p = .0001$). Thus light stress slightly reduced the correlation between zinc content in RPE and choroid melanin granules. This suggests that the light stress altered the metal ion binding capacity of the melanin granules.

Control animals

In RPE and choroidal melanin granules of control animals on the normal zinc diet (n=6 eyes), calcium was the most abundant ion followed by zinc then copper (Table 17 , Figure 55). Low iron peaks were sometimes present in RPE granules but were absent in choroidal melanin granules.

In the animal fed the zinc deficient diet for 11 days (n=2 eyes), PK/BG ratios of Ca, Cu and Zn in the RPE decreased compared to control eyes. The PK/BG ratios of iron increased slightly. In the choroid of this animal the PK/BG of Ca, Cu and Zn all decreased: Cu and Fe were not present above background levels. Feeding the zinc deficient diet resulted in a decrease in zinc level in RPE and choroidal melanin granules but due to the small sample size this difference was not statistically significant (Figure 56 and 57).

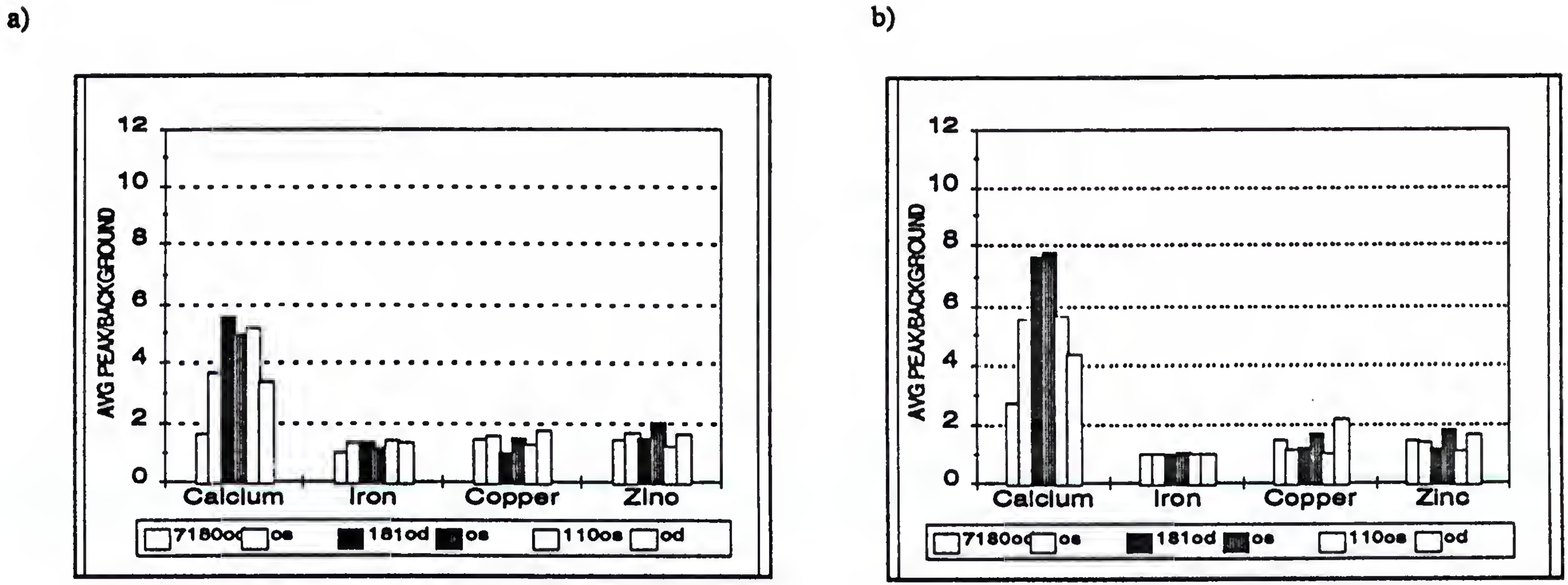


Figure 55: Control animals fed the ZnN diet and not exposed to light. Mean peak/background ratios of Ca, Fe, Cu and Zn in RPE (a) and choroidal (b)melanin granules are shown for both of an animals eyes. A peak to background ration of 1 indicates the element was not present above background. There were three control animals fed the ZnN diet so 6 eyes are shown. Note that the Ca is the most abundant element bound in both RPE and choroidal melanin followed by Cu, Zn and Fe. Iron is not present above background in the choroid. These figures also demonstrate how choroidal melanin typically binds more Ca than RPE melanin.

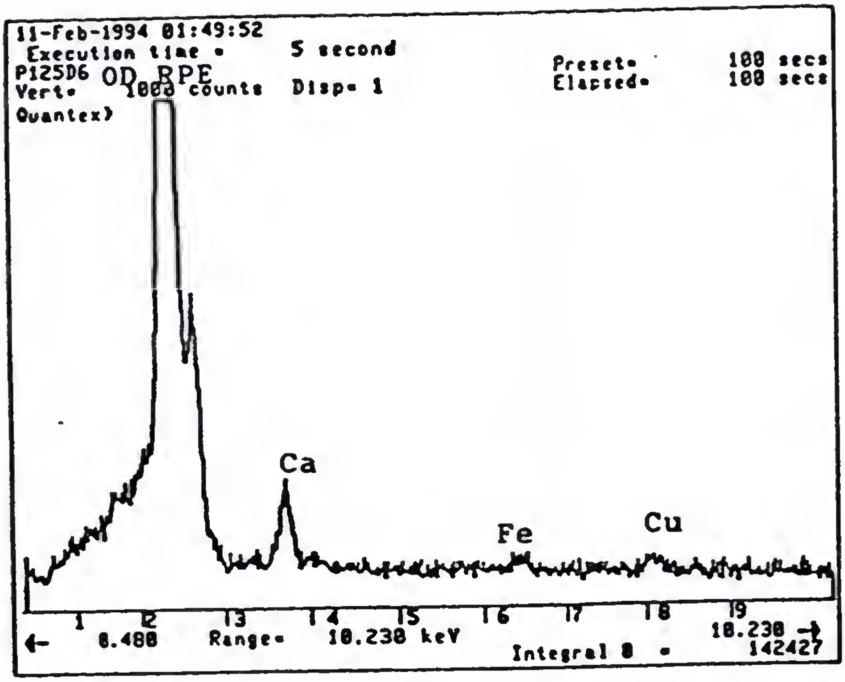


Figure 56: Effect of Zn- diet on elemental spectra. A spectra from the RPE of a control animal fed the Zn- diet is shown. In this case the peaks of Ca, Cu and Zn are less than those of animal fed the ZnN diet. Also note how the Fe peak is still present above background although the other elements are relatively decreased. The increase in iron within RPE spectra was the only statistically significant diet main effect found in elemental spectra.

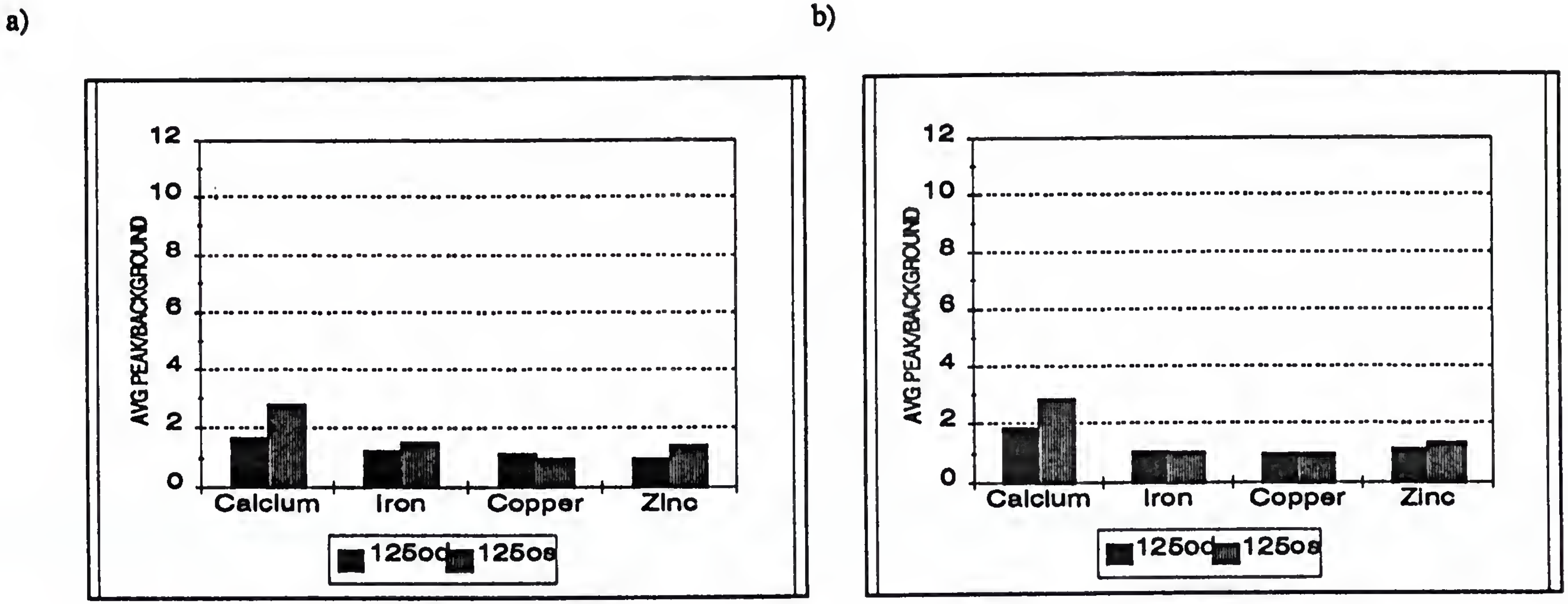


Figure 57: Control animal on Zn- diet. Results from x-ray microanalysis are shown for both eyes for the a) RPE and b) choroid. Note that feeding the Zn- diet appeared to result in decreaesd binding of elements compared to controls. The choroidal melanin in particular seemed to have less Ca and Cu bound when compared animals fed the ZnN diet. Trends must be interpreted with caution since only one animal represents the control.

In the animal fed the Zn+ diet, RPE melanin had lower PK/BG Ca, slightly lower Cu and Zn but iron had a slight increase in PK/BG ratio vs control eyes. In the choroid of this animal, the ion content of the melanin granules were similar to control eyes except for a lower PK/BG ratio of zinc.

Continuous Light Stress (72 hours): 72 hours Post-Exposure

Light exposure resulted in mild to moderate damage in most eyes. A few eyes had severe damage. There was a trend towards more severe damage in the eyes of animals on zinc normal or supplemented diets. In the choroid and RPE of animals exposed to light stress there was a tendency for PK/BG ratios of all elements to increase (Table 17, Figure 58). Figure 59 demonstrates control and light-exposed animals side by side to better show effect of light. The most marked increase occurred in the calcium peaks, especially in the choroid. Calcium peaks often were very high in animals with moderate or severe damage but this did not always occur. Within the three diet groups exposed to light the average PK/BG ratios of zinc were decreased in the eyes of animals on the deficient diet but this decrease was not significant. No correlation between degree of damage and the elemental spectra could be found.

Statistical analysis by 2-factor ANOVA revealed the only significant effect was a diet main effect for RPE iron. The PK/BG ratios of Fe in the RPE of light exposed animals in the different diet groups was Zn- 1.47, ZnN 1.20 and Zn+ 1.26. The ANOVA indicated the means were different with $p = 0.02$ and for the Kruskal-Wallis (KW) $p = 0.05$.

The parametric-test and non-parametric Kruskal-Wallis tests were performed in each diet group comparing control to light exposed eyes. It is apparent that light exposure

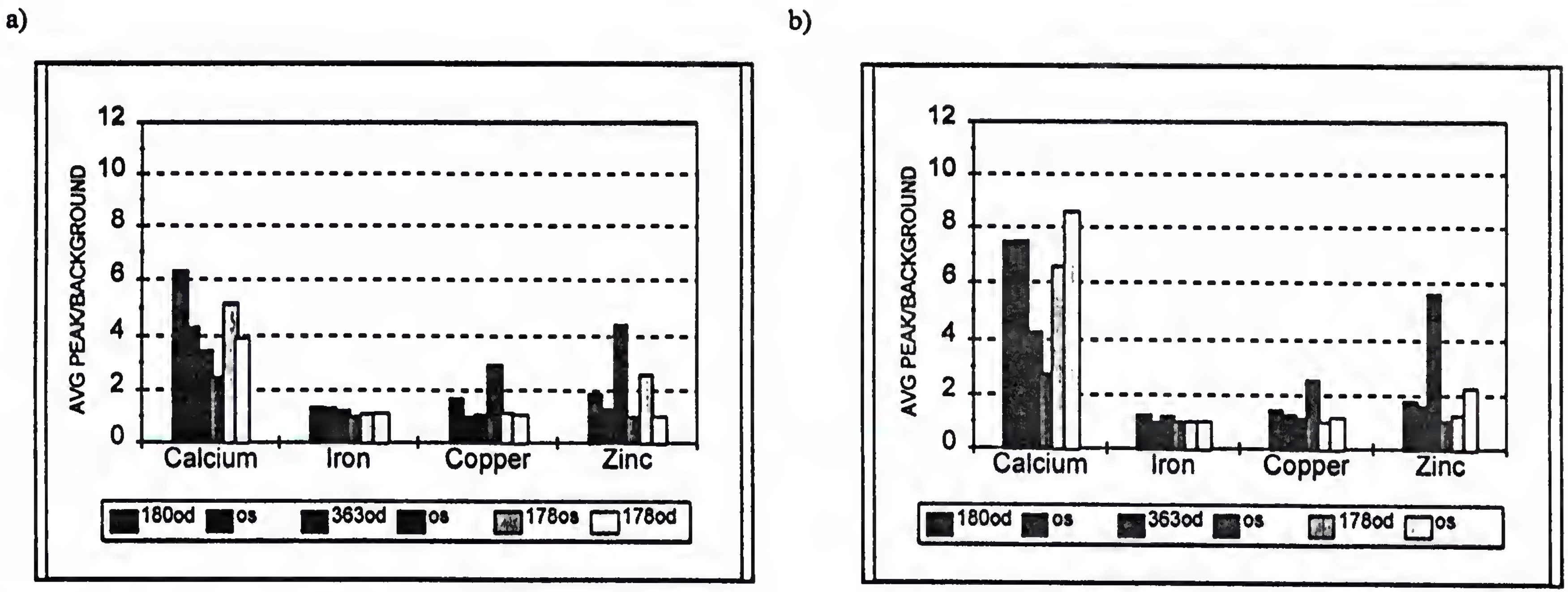
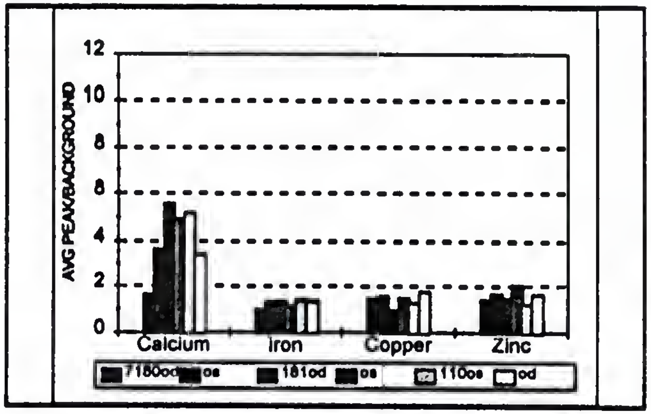
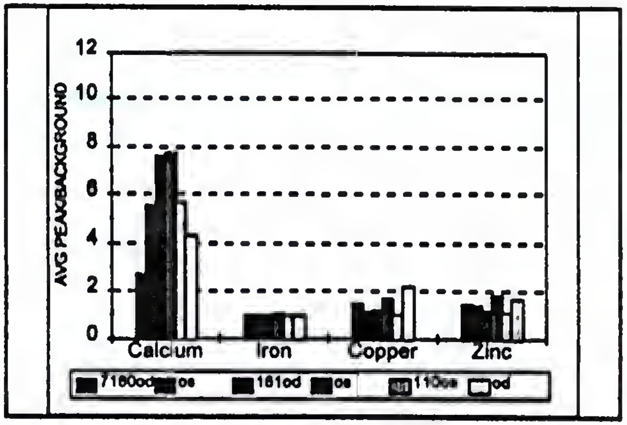


Figure 58: Effect of light-stress on elemental spectrra in animals fed ZnN diet. a) RPE and b) choroid Animals were exposed to 72 hours of continous light and sacrificed 72 hours post-exposure. Note the increase in Ca peaks in the RPE and choroid compared to control eyes. It is apparent from the figures that in animal #363 one eye had very high levels of Cu. In animal #178 that one of its eyes had very high levels of Zn .

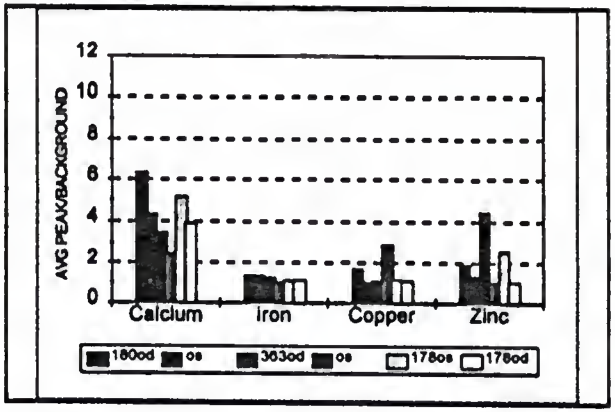
a) RPE of controls.



b) Choroid of controls.



c) RPE of light-exposed.



d) Choroid of light-exposed.

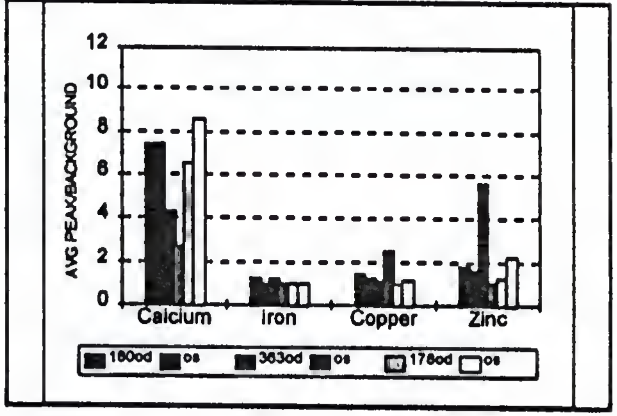


Figure 59: Effect of light on elemental spectra. All animals were fed the ZnN diet. Control and light-exposed animals are shown side by side to facilitate comparison. It is apparent the light appeared to increae Ca bound in many eyes but not in all cases.

a) and b) Mean peak to background ratios from control animals a) RPE and b) choroidal melanin granules. Mean PK/BG ratios from light-exposed c) RPE and d) choroidal melanin granules.

increased the mean PK/BG in the RPE and choroid of Ca in most diet groups; most notably in animals fed the Zn- diet (KW, $p = .18$). In the choroid of animals fed the Zn+ diet the increase in PK/BG ratio of Ca did not occur. Comparison of RPE and choroidal spectra with respect to Ca shows how the choroidal melanin granules appear to have more bound Ca, however, this ability appeared to be decreased in animals fed the Zn- diet. Examination of the spectra with regard to iron indicates that Fe is bound in much smaller quantities than Ca, Cu or Zn, and that RPE melanin binds Fe more than choroidal melanin. As mentioned previously, light exposure and feeding the Zn- diet appeared to increase bound Fe in the RPE and in the choroid. Examination of the spectra with respect to Cu shows a trend towards increased binding of copper in light exposed groups irregardless of diets fed. In unexposed animals it appeared that feeding the Zn- diet decreased the amount of Cu bound to melanin in the RPE and CH (KW, $p = .09$). Examination of the spectra with respect to Zn shows that in unexposed animals feeding the Zn- diet did result in a slight decrease in bound Zn. Feeding the Zn+ diet however did not significantly increase the amount of Zn in the melanin granules. Again with regards to Zn bound to melanin there was a trend toward increased binding of Zn as a result of light exposure.

It is apparent that continuous light exposure for 72 hours in general resulted in increased metal ion binding by choroidal and RPE melanin granules. This increase was greatest for calcium. Feeding the Zn- diet appeared to induce a loss of all elements bound to the melanin granules when compared to data from the eyes of control animals.

Three Weeks on Experimental Diets: Light Stress and Controls

In this group only one animal (2 eyes) was analyzed for each data point so statistical analysis was not performed. Table 18 summarizes the elemental spectra for control and light-stressed animals maintained on their experimental diets for three weeks. In the control animal fed the diet for 3 weeks no remarkable alterations in the spectra from the other control animals was observed. There was some asymmetry between the RPE and choroidal elements between the two eyes with one eye having low levels of Fe, Cu and Zn. This eye histologically had some evidence of prior damage and recovery so the significance of this asymmetry is unknown. The animal exposed to light and then maintained for 3 weeks after exposure showed increased calcium levels in both eyes compared to the control animals. In the RPE the amount of iron bound also appeared to increase, while the copper and zinc levels did not differ from the controls.

In the control animal fed the zinc deficient diet for 3 weeks there was a marked variation in elemental content of RPE melanin in the two eyes. In one eye Ca and Fe mildly increased. Copper and Zn peaks were similar to control eyes. The opposite eye had a paucity of ions with only moderate Ca and Zn peaks. The amount of zinc bound did not decrease dramatically after feeding the zinc deficient diet for 3 weeks. The choroidal melanin granules in the control animal in one eye had a significant increased in Ca in one eye while the other eye's Ca peak was not different than that of the control. Again the level of zinc was fairly high considering the animal was fed a zinc deficient diet for three weeks. In the animal fed this diet and exposed to light, elemental content of RPE melanin again varied between eyes and zinc levels decreased in one eye and increased in the other.

Table 18: Summary of mean PK/BG ratios of elemental spectra from eyes of control and light-stressed animals (72 hours of continuous light) maintained on their diets for three weeks after exposure. The standard deviations (std) of means are also shown.

	RPE MELANIN - element								CHOROID MELANIN - element							
	Ca	std	Fe	std	Cu	std	Zn	std	Ca	std	Fe	std	Cu	std	Zn	std
Controls																
ZnN 361od	2.69	0.23	1.12	0.14	1.13	0.16	2.66	0.47	3.77	0.70	1.09	0.12	1.47	0.15	3.39	0.54
361os	1.73	0.17	1.07	0.15	1.00	0.06	1.00	0.00	3.08	0.59	1.00	0.00	1.47	0.15	1.00	0.00
Zn- 362os	2.86	0.81	1.18	0.24	1.50	0.17	3.57	1.32	3.21	0.68	1.28	0.41	1.30	0.07	2.77	0.51
362od	4.61	1.12	1.15	0.25	1.06	0.12	1.85	0.25	9.48	1.52	1.00	0.18	1.02	0.17	2.47	0.25
Zn+ 366od	5.42	0.73	2.98	3.60	2.55	0.95	2.50	0.98	8.24	2.12	1.03	0.08	2.49	0.92	2.64	0.97
366os	1.94	0.37	1.11	0.15	1.11	0.17	3.22	0.74	2.37	0.33	1.04	0.11	1.16	0.13	5.10	1.33
Light Stress																
ZnN 110os	5.17	0.74	1.41	0.42	1.28	0.19	1.24	0.29	5.63	0.87	1.00	0.00	1.03	0.26	1.11	0.14
110od	3.40	0.33	1.36	0.09	1.75	0.21	1.64	0.10	4.32	0.78	1.00	0.00	2.19	0.54	1.66	0.17
Zn- 111od	1.74	0.15	1.00	0.00	1.01	0.14	2.19	0.46	2.24	0.65	1.00	0.00	1.04	0.29	2.48	0.74
111os	4.02	1.10	2.17	1.39	1.38	0.17	1.23	0.24	6.74	0.98	1.13	0.15	1.22	0.29	1.16	0.20
Zn+ 109os	4.80	2.00	1.18	0.22	1.18	0.17	1.60	0.17	7.98	3.14	1.26	0.20	1.26	0.18	1.60	0.12
109od	4.37	0.86	1.00	0.23	1.19	0.26	1.38	0.23	5.19	1.22	1.00	0.00	1.21	0.08	1.42	0.41

In the control animal fed the Zn+ diet (fed diet from Part 1 of the experiment, 300 ppm Zn) for four weeks one eye appeared to have increased zinc levels while the other had levels not different from those fed the ZnN diet. In one eye there was an increase in Ca in the RPE and choroid while the other did not exhibit this increase. The spectra from the left eye of this animal demonstrates how the level of Ca and Zn bound to granules often exhibited an inverse relationship (Figure 54). In the animal that received light stress and was fed the Zn+ diet, there was an increase in all elements in one eye while the other eye had a decrease in all elements except zinc which increased. In this case feeding the Zn+ diet (240ppm Zn) did not seem to influence the amount of zinc bound to ocular melanin granules.

CHAPTER 5
DISCUSSION

Porcine Animal Model

The pig represents a useful and anatomically similar animal model to study retinal disease in humans. The pig has a holangiotic retinal vascular pattern which is similar to humans. The pig retina also contains numerous cones, an area centralis, and lacks a tapetum. The larger eye size of the pig increase the amount of tissue available for analysis. Considering all these factors, when compared to common laboratory species such as the rat and mouse, which have pure rod retinas and small eyes, the pig maybe a more useful animal model for the human eye.

The results of this study suggest and clarify statements made by other authors regarding the cone content of the porcine retina.[22-24] In Beauchemin's paper, the author commented on the relatively even distribution of cones throughout the porcine retina, but no photoreceptor counts were made.[24] This study utilizing photoreceptor counts of one micron histologic sections has shown that the pig retina possesses a cone rich area centralis in the superotemporal retina. The rod to cone ratio of the superotemporal quadrant changes from around 8-9:1 in the peripapillary region to 4-5:1 in the area centralis and then increases again in the periphery to 5-6:1. It is apparent from these values that rather than being a circular area in the superotemporal fundus, the area centralis is more of a horizontal oval. This finding is similar to another author's findings in which histologic and gross examinations led him to conclude that the area centralis of the pig was a horizontal streak in the posterior pole.[188] De Schaepdrijver also mentioned that cones are relatively evenly distributed and numerous, but that they decrease in the periphery and are more concentrated in the central area.[25] Another study found a statistically significant difference in density of photoreceptors between the areas centralis and the peripheral retina with mean density of 82 receptors /mm in the peripheral retina and 172 receptors /mm in the central retina.[189] The findings here substantiate the abundance of cones in the porcine retina but do show that the distribution is not even. The mean rod to cone ratio in the superotemporal quadrant was 5.5:1 and in the inferotemporal quadrant was 6.7:1 again demonstrating the increased density of cones in the superotemporal quadrant. If the pig's popularity as an animal model for eye diseases continues to increase the nuclear density and rod to cone ratios of all retinal areas should be determined.

In this study the nuclear density was determined in the central retina of the superotemporal and inferotemporal quadrants and was found to be approximately 25,000 cells / mm^2 with counts sometimes being as low as 20,000 or as high as 30,000 cells / mm^2 (see Tables 10-12). These values are similar to the results of the only other study on photoreceptor density in the porcine retina in which they found 29,500 receptors / mm^2 in the central retina and 6,700 receptors / mm^2 in the periphery.[189] This density of photoreceptors is much less than that found in the primate retina. In the Cynomolgus monkey, for example, the parafoveal rod density was around 147,000 cells / mm^2 and the cone density 12,000 for a total cell density of around 160,000 cells / mm^2.[187] Although cones are abundant in the porcine retina and the pig retina has a relatively avascular area centralis in which they are more concentrated, there is not a true avascular fovea in which only cones are found. In the Cynomolgus monkey the fovea possesses only cones and their density ranges from 150,000 to 200,000 cells / mm^2. Even though the porcine retina does not possess numbers of photoreceptors as high as the primate, the relatively large number of cones compared to the rat or other laboratory species enables better extrapolation from results of porcine studies as to how the human retina, particularly the macula and fovea with their high cone density, might respond.

Another advantage of the pig is the size of its eyes which makes more tissue available for experiments. Also, the pig is a less expensive animal model to utilize than primate species. Obviously primates represent the most anatomically correct animal model to study human eye diseases, but the ethics and expense of utilizing this species can be troublesome.

There are also disadvantages of utilizing the pig as an animal model. Pigs are large, and more difficult to handle and anesthetize compared to a smaller laboratory animal such a the rat. For light studies involving free-ranging animals, special units must be designed to deliver high intensity light which is can be technically difficult and cost prohibitive. For nutritional studies the pig is a good choice since it a monogastric omnivorous species similar to man. The pig also has similar zinc metabolism to that of man. However, special facilities may be necessary to provide housing, water and special diets in studies in which zinc deficiency is trying to be induced.

Zinc Status

The statistical analysis of variables thought to be indicative of zinc status (serum, liver and kidney zinc, and kidney metallothionein) revealed that the zinc status of the experimental animals was not significantly altered. Statistical analysis of animals from Part 1 (intermittent light exposure) was not possible because there was only one animal per data point. Since 3 animals were maintained on each diet and light group in Part 2 of the experiment (72 hours continuous lighting), statistical comparisons between the three groups could be performed, but still no significant alteration in biochemical parameters occurred as the result of the different dietary levels of zinc.

In addition to the ocular anatomical similarities to the human, the pig was employed as an animal model because abundant data is available on zinc metabolism in this species.[127,134,141,152-155,190] The classic signs of zinc deficiency in pigs are reduction in growth rate and food intake, low plasma zinc concentration and clinical signs of parakeratosis.[152-154] In one study piglets were fed an experimental diet of 12 ppm zinc and a control diet with 90 ppm zinc to investigate the effects of zinc deficiency on tissues in pigs.[155] Pigs maintained on the zinc deficient diet exhibited growth retardation within 2 weeks of being fed the diet. Feed efficiency was half that of the control pigs, and parakeratosis was moderately severe. The serum concentrations of zinc also decreased significantly compared to controls. Other studies have shown a similar significant decrease in plasma zinc concentration after feeding a diet with 25 ppm of zinc for 10 days, i.e., the serum zinc concentration decreased from 65 µg Zn/ 100ml to 46.1 µg Zn/ 100ml.[152]

In our study no decrease in serum zinc levels were observed in animals fed the zinc deficient diet and no increase was observed in the animals fed the supplemented diet. The animals on the zinc deficient diet however, did exhibit decreased weight gain in terms of kg/ day which suggests there might have been a mild negative zinc balance. At the time of sacrifice, the experimental animals had been on the zinc deficient diets for 11-13 days and yet only a few exhibited a substantial decrease in serum zinc compared to baseline levels. If any systemic negative zinc balance was indeed present in our pigs, it was mild.

Numerous physiological factors have been shown to influence circulating zinc levels including short term fasting, food intake, circadian variations, stress, and tissue necrosis.[106-108,129] Infectious or inflammatory stress will mediate a major, acute sequestration of circulating zinc into the liver with a decline in plasma concentrations.[107,108,129] An example of the pitfalls in determining zinc status has been the finding in studies with human subjects that some patients had stable plasma/ serum zinc values despite a systemic negative zinc balance when assessed by other methods.[129,131] Other factors such as external contamination of glass or plastic containers, hemolysis, or prolonged venous occlusion may elevate zinc levels.[107,108,129] Since all of these factors affect serum zinc levels, it cannot be utilized as the sole assessment of zinc status. Therefore, in this experiment liver and kidney Zn and metallothionein content were also determined.

In contrast to serum or plasma Zn levels, expression of the metallothionein (MT) gene is proportional to zinc intake.[106] Serum or red blood cell MT levels determined by radioimmunoassay or ELISA represent superior methods to assess zinc status in humans, but these assays are not available for the pig. Studies on levels of MT in the liver and kidney of rats exposed to various stresses revealed that the kidney MT level was much less responsive to various stresses (i.e. heat burn, strenuous exercise, CCl_4) than the liver.[182] In the present study to provide a better assessment of zinc status, evaluation of the kidney and liver MT levels was performed on all pigs in addition to the tissue zinc content. In some cases trends of increasing tissue zinc content with increased dietary zinc level were observed. Examination of kidney MT levels also revealed a trend towards lower MT levels in the Zn- fed animals but this was not statistically significant.

The lack of changes in serum and biochemical (i.e. liver, kidney Zn and MT) values and the lack of symptoms such as parakeratosis or dermatitis suggested our protocol did not induce a significant negative zinc status. Even in the animals fed the zinc deficient diets for three weeks, dramatic clinical or biochemical changes indicative of zinc deficiency were not evident which suggests that an exogenous source of zinc may have been ingested by these animals. This was not surprising in the first part of the experiment because of the housing problems which allowed the pigs intermittent access to the galvanized chain link on the cage which may have been a source of exogenous zinc. In the second part of the study, it was more surprising that the Zn- diet did not alter the status of the animals because the animals were housed in stainless steel cages. It is possible that, even in part two of the experiment, the pigs somehow found a source of exogenous zinc. The water may have been contaminated during transport in plastic containers or at the source. Also, during cage cleaning when pigs were allowed to roam in small enclosure for a short period, they may have found an exogenous source of zinc. The water was delivered through silastic tubing and a lixit dispenser which may have contained alloys possessing zinc.

Alternatively, these young pigs may have had sufficient zinc reserves within their tissues i.e. liver and bone, which were able to prevent a significant alteration in zinc status. These tissues are potential storage sites of zinc.[108,129] The concentration of zinc in bone is quite high and it may be released when bone resorption occurs.[108] In these young pigs, still in the growth phase, bone deposition and resorption are occurring so the bone zinc stores may have prevented a rapid decrease in the zinc status. It is more likely that the liver may have had substantial stores of zinc that could have prevented or buffered against a rapid onset of a significant negative zinc balance. Studies have shown that in the pig, the level of zinc in the liver at birth is three- to fivefold higher than in the adult, and it falls rapidly in the first 3 -4 months postnatally.[108]

The average kidney MT for the animals fed the low, normal and high zinc diets revealed that the animals fed the low zinc diet had lower MT levels. There was no difference in MT levels between the groups fed the normal and high zinc diets. The kidney and liver zinc only occasionally revealed a similar trend. Most likely, the low and high zinc diets were not fed long enough to induce significant changes in zinc status.

If additional studies are performed using the porcine animal model, the protocol to induce zinc deficiency and supplementation will need to be altered. Much more rigid control of food intake, prevention of access to exogenous zinc and a longer period of feeding the zinc deficient diets must be utilized. Confirmation of induction of a zinc deficient state should be done before exposing the animals to the light stress. For example, serum zinc and alkaline phosphatase activity could be used as indicators of any effects of feeding a zinc deficient diet. In addition, the assays available to determine human serum and red blood cell

MT could be modified for use in the pig. As important as biochemical data, clinical signs such as parakeratosis, decreased appetite and growth retardation, compared to a pair-fed control group should be present before exposure of animals to the light stress. Finally, a more balanced experimental design with equal numbers of control and light-exposed animals and a greater number of animals per each group should be utilized. This pilot study has provided insight into how future studies might be designed. However, because feeding the different diets did not significantly alter the zinc status of animals in this study, the effect of feeding the diet on the light induced retinal injury must be, and will be, interpreted with great caution.

Photic Injury

The purpose of this study was to utilize the pig as an animal model of light induced retinal damage and then to determine how feeding high or low levels of zinc in the diets alters light induced damage to the retina. Nutrition and cumulative light exposure are two suspected etiologic factors important in the pathogenesis of age-related macular degeneration in humans (ARMD). Since a major goal of this study was to add to the understanding of the multifactorial etiopathogenesis of ARMD, it was decided to try to induce injury in unanesthetized free ranging animals by housing them in an environment with high intensity light. This was felt to be a more natural type of light exposure that more closely mimics the type of light exposure affected humans might receive.

The desire for even distribution of bright light in the environment led to the design of a circular unit with white formica walls. Because the unit had to be elevated off the floor to allow for ventilation, cooling and cleaning, the animals were able to partially shade at least one eye at a time by pressing one side of the head against or partially under the edge of the wall. This could have contributed to the multifocal distribution seen within some eyes and also the asymmetry of damage between eyes of some animals. It is possible that an animal may have alternated shading of each eye for similar periods of time, or an animal may have shaded one eye more than the other, for example, if an animal preferred to lay on one side or the other. Shading behavior was observed in most animals while they were in the unit but the amount of time that each eye was protected was not recorded. Future studies would require modifications to the unit or construction of a unit that would prevent shading behavior.

The initial pilot study indicated light damage could be induced in the porcine eye. The hyperthermia that resulted from the incandescent light sources suspended over the small area of the cage however accentuated the photic injury. Both eyes of both animals (one exposed to 36 and the other 72 hours of intermittent light) were severely damaged. The damage was characterized by diffuse ONL and occasional INL chromatolysis, and retinal edema. This pilot study retrospectively led to the conclusion that photic injury in the porcine animal is accentuated by heat stress. This has been shown in other animal models of photic injury such as the rat and mouse.[2,39,43,191,192] In rats for example, with a rectal temperature of 36.4°C, 48 hours of continuous broad band green light exposure was required to induce severe degeneration of the retina.[192] With rectal temperatures of 40°C an exposure time of 10 minutes with the same light intensity induced a similar retinal degeneration by an almost 300 fold reduction in exposure time. The explanation of how the temperature affects in vivo light damage experiments probably lies in the speeding up of metabolic processes. In these experiments the extra variable of heat stress was not desired, so in the main study the unit was adapted so that animals in the unit did not experience hyperthermia. The subsequent studies utilizing intermittent light however, had much less damage than that found in the pilot study. The difference in damage between the hyperthermic and normothermic animals, however, show that in the porcine photic injury model hyperthermia accentuates retinal damage.

Part 1 of the study investigated the effects of 36 and 72 hours of intermittent light stress on the porcine retina without heat stress. Part 2 of the study utilized continuous light stress to enable comparison between intermittent and continuous light exposure. In the continuous light group, to facilitate interpretation of results, three animals or 6 eyes were in each data group (all received light, 3 groups of 3 pigs each received different dietary zinc levels) to allow statistical analysis of the results. In both schedules of light exposure, one animal on each of the three experimental diets was maintained for three weeks post-exposure to observe chronic changes if present. Due to financial constraints, the number of control animals had to be limited and some were utilized as controls for both Part 1 and 2 of the study.

Two types of damage were observed in the intermittent and continuous light exposure groups. The first type of damage involved multifocal nuclear death or chromatin alterations confined to the ONL. Only the occasional OS, RPE cells or INL cells were damaged. The other type of damage involved diffuse retinal edema, multifocal nuclear pyknosis or chromatolysis in the ONL and to a lesser extent the INL, and inner and outer segment disruption and degeneration. The RPE often was necrotic or edematous and in some cases had sloughed off Bruch's membrane. The milder type of damage was more common in the intermittent light-stress group and the severe type of damage more common in the continuous light-stress group. In the intermittent light group, there was a tendency towards a dose response in that 3 of 6 eyes exposed to 36 hours of intermittent light had significant damage compared to 4 of 6 eyes exposed to 72 hours of intermittent light.

In the mild type of damage, changes in nuclear chromatin which were visible at the light and electron microscopic level were notable. Alterations in nuclear chromatin included rod nuclei becoming more euchromatic with rounding up of the nucleus or rod and cone nuclei exhibiting pyknosis. The mild alterations in ONL nuclear chromatin were also observed in some of the control eyes. Even in control retinas there were areas where a relatively high percent of the nuclei (i.e. 5%) had this appearance. This type of change was rarely observed in the cones which lie most external in the ONL, just inside the external limiting membrane. However, due to the euchromatic nature of normal cone nuclei it would be difficult to detect. Whether or not this alteration represents a pathological change remains unclear. It is possible that these cells were not undergoing degeneration but rather they represent cones that had redistributed within the rods rather than resting in their normal position on the ELM. The only way to definitively determine the nature of these cells would be with immunohistochemical techniques that allow differentiation of rods and cones.

When nuclear pyknosis was apparent, profiles of degenerating nuclei and their associated inner segment (IS) became compact and stained densely. At the electron microscope level the IS morphology was also obviously abnormal compared to normal healthy IS. Specifically the mitochondria were swollen, loss of cristae was evident, and in severe cases the IS was filled with homogenous debris with no identifiable mitochondria. When the nucleus and its associated IS were pyknotic, the nucleus in most cases had displaced outward beyond the ELM towards the OS layer.

Degenerating cells, i.e., pyknotic with movement through the ELM, were observed in control eyes as well as in those exposed to light, but with much less frequency. Some cell degeneration occurs in the normal maturing retina.[25] This is believed to occur in cases in which cells proliferated excessively and/or failed to match with other neuronal cells for future synaptic connections. Studies have shown the number of nuclei decrease in the rat and pig retina as they mature.[25,193] Studies on the developing porcine retina have shown it is nearly mature by one week before birth, and the retina slowly becomes thinner in the first six months post partum. This is mostly due to a increase in the surface area of the enlarging globe upon which the immature retina must spread over and therefore thin out.[25] The pigs in this study were 6-8 weeks of age so the occasional dying cell could be considered normal, but when the frequency increased it probably was due to the light stress. However, in some eyes in which only one focus of a few dying cells were found in several sections, the change could not confidently and therefore was not attributed to light-stress.

In contrast to other photic injury studies, in this experiment, the outer segment (OS) of the pig appeared to be resistant to photic injury.[31,34,35,39,194,195] Outer segment disruption, as an early histologic change during photic injury, is the hallmark of Noell's type II retinal damage in rats.[39] Minimal OS disruption was present in the eyes that exhibited multifocal dropout of nuclei. Only the obviously dying cells exhibited degenerating OS. It was notable that in the pig retina, the IS appeared to be more sensitive than the OS. Even in mildly damaged eyes several of the IS mitochondria were degenerative. One study utilizing rats and a 15 minute exposure to blue light showed that the IS is adversely affected by light as the result of an immediate reduction in cytochrome oxidase activity .[70] This suggests that the mitochondria are very susceptible to light-stress and partially explains the apparent sensitivity of the IS of the porcine retina to light.

The lack of significant OS disruption in the mildly damaged porcine retinas of this study may simply substantiate the fact that the light-stress in this experiment was below the threshold to induce damage in the majority of retinal cells. The OS certainly exhibited damage in the more severely damaged eyes in which all layers of the retina were affected. In the one light exposure study that has been performed in piglets, mild OS vacuolation was found after 72 hours of continuous exposure to 300 foot-candles of blue fluorescent light.[30] In this study 600 foot-candles of broad spectrum incandescent light was the light source. The disruption of OS from blue light exposure was relatively mild, and as in our study multifocal nuclear pyknosis of the ONL layer was the only other accompanying histologic change suggesting mild damage had been induced. The blue light most likely induced more disruption of the OS compared to our broad spectrum white light because of its higher energy. Several studies in monkeys and rats have shown that the threshold for light damage increases exponentially with decreasing wavelength.[40,41,43,44,52,196]

The two types of damage observed in the intermittent light study also occurred with the continuous light exposure. Continuous light exposure seemed to result in more severe damage in a greater number of eyes than the intermittent light-exposed group. The more severe type of damage that affected all retinal layers, including the RPE, often was observed in this group, but was observed only once in the intermittent light-exposed animals. Notable was the markedly adverse effect the continuous light-stress had on the RPE layer. Numerous cells in the RPE layer exhibited several of the following changes: vacuolation of smooth endoplasmic reticulum and cytosolic edema, irregular and darkly staining pyknotic nuclei, nuclear chromatolysis, detachment from Bruch's membrane, and weakening of the RPE microvilli interdigitations with the OS. It follows that, in eyes in which RPE cells were detaching from Bruch's membrane, breakdown of the blood retinal barrier had occurred. Studies on rabbits and monkeys indicate that breakdown of the blood retinal barrier is one of the first changes observed in light damage but is best detected by fluorescein angiography.[3,197] Fluorescein angiography may have revealed breakdown of the blood retinal barrier in these eyes and in future studies would be a useful adjunct diagnostic tool to identify photic injury.

In most, but not all cases, RPE damage usually occurred in retinas in which all layers seemed abnormal, i.e., INL, IPL layers being edematous and all nuclei of ONL appeared abnormal. The marked destruction of the RPE bears similarity to Noell's type II retinal damage described in rats.[39] In this type of damage the RPE is destroyed in association with destruction of rod nuclei in the ONL. Outer segment disruption is also prominent. All of these findings were observed in the severely damaged porcine eyes. Retinal pigment epithelial layer destruction was also observed by Tso in monkeys exposed to the incandescent light of the ophthalmoscope.[3,33,198] It appears that incandescent light, with its broad spectrum light containing both short and longer wavelengths, is particularly damaging to the RPE layer.

Within light exposed eyes, subjectively, it appeared that light exposure resulted in a slight increase in number of lipofuscin granules compared to control unexposed eyes. The numbers of lipofuscin granules however were not very high which can be attributed to the young age of these pigs and also to the relatively short duration of light exposure. Also, most of the eyes were harvested 72 hours post-exposure and lipofuscin formation would not have occurred this rapidly. The few animals that were sacrificed 3 weeks post-exposure did have more numerous lipofuscin granules but again there was not a great difference in abundance between controls and light treated animals. Several mildly osmiophilic homogeneous granules were observed in the apical cytosol of RPE cells. This type of granule could represent a primary lysosome, a secondary lysosome with well digested material, or lipofuscin.[87] Lipofuscin granules, a type of secondary lysosome, typically have undigested membranoglobular material and are much more osmiophilic.[87,187] Autofluorescent microscopy would be required to determine if light or dietary zinc influences the lipofuscin content of the RPE.[87,88] Alternatively, acid phosphatase staining would allow ultrastructural identification of lysosomes to confirm that these granules are lysosomal rather than lipofuscin.

In other photic injury studies lipofuscin accumulation is not a remarkable feature.[43] In Japanese quail, a single high intensity 18 hour light exposure in 4 month old animals did not result in any increase in lipofuscin when eyes were evaluated 6 weeks post-exposure. However, a second exposure at 12 months with subsequent examination 6 weeks post-exposure did reveal an increase in lipofuscin compared to controls.[203] It would require more long term studies with older animals to see if increased lipofuscin formation occurs in the light stressed eyes of pigs.

As far as the effect of dietary zinc on lipofuscin, none was observed in this study. This is not surprising since the diets were fed for a short period, the animals were young, and also zinc status was not significantly altered. All of these factors make it unlikely that RPE lipofuscin content would change as a result of dietary zinc level in this experiment. Samuelson et al. found an increase in lipofuscin occurs with age in the young porcine eye and subjectively, the authors felt that the low zinc diet accentuated this increase.[21] In that study however, pigs were fed the low zinc diets for 8-13 months. In the present study, the diets were fed for a much shorter period, 10 days to 4 weeks, so it is not surprising no marked alteration in lipofuscin content was noted in the RPE. Results of a study by Aouididi et al. substantiate this finding. Four and seven year old sows were fed a low zinc diet for 6 months and fluorescent microscopy performed on the RPE layer. Lipofuscin in the pig RPE was much lower than that found in aged human eyes. The investigators did not find any increase in lipofuscin in the eyes of the zinc deficient animals.[202] These older animals however, probably had much more abundant granules than the younger animals used in this and Samueson's study which may in part explain why low dietary zinc level may not have significantly affected the liopofuscin content of the RPE.

The two types of light damage observed in porcine eyes may also represent a time course in degeneration that can vary between individuals. The diffuse retinal edema and OS changes observed in some eyes may have been an early change that was transient, and then if damage was sublethal and recovery occurred, all that was observed was the loss of the few cells that were irreversibly damaged, i.e., the multifocal dropout of ONL nuclei observed in several eyes.

A brief discussion on the different types and degree of damage in the intermittent vs. continuous light exposed group is warranted as this finding has been described previously. Organisciak performed studies on rats with 200-250 foot-candle green light delivered in continuous or intermittent doses. Twelve 1-hour-light, 2-hour-dark exposures were compared to 24 hours of continuous exposure. Intermittent light exposure resulted in greater damage than continuous exposure.[201] His intermittent light schedule, however, differed markedly from this study in which 18 hours of light was followed by a dark cycle of 6 hours. Noell, in his study on rats, found a similar cumulative and greater damaging effect of intermittent light when the dark periods were 6 hours compared to continuous exposure, but if the dark intervals were 24 hours or greater, no retinal damage occurred.[39] In another study, rats exposed to 24 hours of continuous or intermittent (three 8 hour exposures with 7 days of dark) 150-175 foot-candle green fluorescent light, more damage was observed in the continuously exposed group.[27] In monkeys intermittent blue and green light produced damage to blue and green cone cells while continuous exposure created more severe damage to the RPE.[202] It appears that the length of the dark period influences the degree of damage that occurs with intermittent light. Repair mechanisms in the retina are probably able to compensate for the damaging effects of intermittent light exposures when longer intervals between exposures are allowed. In our case the 6 hours of dark probably allowed the retina to recover better before the next onset of light. In addition to the intermittent light schedule, the difference between this and other studies may lie species differences in retinal cone content, differences in light intensity and wavelength, and other species differences, i.e., the transmissibility of different wavelengths of light by the cornea and lens of the eye.

Organisciak found that intermittent light adversely affected the rat RPE more than continuous light, again differing from the porcine eye in which the continuous light most definitely induced RPE degeneration.[201] Lee found that the rat RPE was well preserved in his intermittent light group compared to the continuously exposed group in which RPE cells were often necrotic.[27] The intermittent light schedule differed markedly between these two studies with Organisciak utilizing alternating 1 hour light-2 hours dark and Lee utilizing 8 hours of light with a 7 day dark interval. As mentioned above, continuous light exposure in the monkey resulted in more severe damage to the RPE, similar to that seen in pigs.[202]

An important drawback of this study was the individual variation in degree of damage between animals of the same test group and within eyes of the same animal. For example, in the animals exposed to 72 hours of intermittent light, one had severe damage in both eyes with diffuse retinal edema, ONL and INL chromatolysis, and OS and RPE degeneration. Another animal had the other type of injury characterized by multifocal dropout of ONL with relatively normal RPE, OS and inner retinal layers. In the other animal the retina was normal with only focal areas of mild damage. The variation in degree of retinal damage made it difficult to document any effect of dietary zinc level on photic injury to the retina.

As occurred in the intermittent light group, the amount of photic injury induced in the continuously light-stressed group was sporadic between individuals, their eyes, and highly variable in characteristics and degree. This type of variation within and between the diet groups suggests that the dose of light utilized must be near the porcine retinal threshold for photic injury.

A major problem was the "multifocal" nature of the damage. Central and more peripheral sections of the wedges were examined for damage. In only 5 of 9 light-exposed eyes definitive retinal damage was present in both of these sections. In eyes with only focal areas of damage it is questionable as to whether or not the focal areas of damage were induced by the light. These focal areas of pyknotic cells or the rods with altered nuclear chromatin pattern and rounding up of the nucleus represent degenerative areas. These foci may have been light-induced. It is also possible that they represent an artifact of razor cuts but if this were the case they would only be at the end of sections where cuts were made and this was not usually the case. They might have been focal areas of chorioretinitis; either active or resolving infectious degeneration (i.e. virus or bacteria), or associated with a non-infectious process such as vasculitis or endotoxemia. However in either of the later suggestions an inflammatory response of some type should have been present and inflammatory cells were not found in any of the foci of degneration.

The multifocal nature of the damage most likely is a manifestation of the subthreshold nature of the light dose, in that some, but not all cells exhibited damage microscopically. The few animals examined electroretinographically and morphologically 3 weeks post-exposure did not exhibit significant retinal damage which, substantiates the mild to moderate degree of damage that must have been induced by the light stress. In several of these eyes however, displaced normal appearing nuclei were observed in the IS layer. Also, normal appearing IS were commonly found on the surface of the RPE. These findings suggested that degenerative changes had occurred due to light, but also, that cellular recovery had occurred. This finding, along with the lack of significant decease in ONL density or thickness, confirm the mild nature of any light-induced retinal damageproduced in this experiment.

It is possible that even though morphological damage appeared multifocal and/or mild to moderate in most cases, more sensitive indicators of cell damage such as fluorescein angiography or histochemical staining for damaged DNA or apoptosis may have revealed more diffuse cell damage. In early light damage these types of changes may be occurring while the retina appears normal on histologic examination.[197,203,204] Apoptosis is associated with physiological or programmed cell death, in contrast to necrosis which is associated with cell injury.[205] Apoptosis for example occurs during embryonic development and in cell systems responding to mild thermal or metabolic stress. Photic injury in rats induces apoptosis.[203] Furthermore, both intermittent and continuous light exposure in rats induced apoptosis with intermittent light inducing it earlier and more extensively than continuous light of similar intensity and exposure dose.[206] Gross features of apoptosis identified by electron microscopy include nuclear chromatin condensation, compactness of cytoplasmic organelles, and the appearance of pedunculated protuberances on the cell surface.[205] Apoptotic cell appearance, however, is limited to only a few minutes, so without histochemical techniques it is difficult to observe. Other features of apoptosis in cell systems are, the deletion of single cells, compared to groups which occurs in necrosis, the lack of inflammatory response, again in contrast to necrosis, and phagocytosis by adjacent cells more than that by macrophages which typically dispose of pyknotic cells.[189] In the light damage induced in the porcine retina the multifocal alterations in nuclear chromatin may have been the result of apoptosis. However ultrastructural examination of such nuclei did not reveal cell membrane blebbing which is typical of apoptosis.[205]

Examination of photographs of electron and light microscopic changes induced by light in primate and human eyes reveals a remarkable similarity in the type of damage.[3,32,35,40,44,52,198,207] The similarity between the porcine and primate to each other is much greater than either bears to the rod dominant rat retina in which many photic injury studies have been performed. Particularly, the histologic appearance of degenerative cones displacing through the ELM is remarkably similar to that found in primate eyes exposed to light.[198] Although the light stress in this study did not induce the classic type I and II damage as described by Noell, a damage very similar to his type II was induced by continuous light. Thus, the pig represents a useful animal model in which to undertake further studies on light damage. It is more practical to utilize than primates, and among the domestic species used as animal models, the pig possesses a large number of cones that are concentrated in an area centralis. Developing a porcine retinal light damage model will be especially useful to study the effects of light on cone photoreceptors. For example, it has been shown that the porcine retina possesses red, blue, and green cones similar to the human eye.[26] This similarity would enable use of the pig retina to study how the various types of cones are affected by different intensities, schedules, and/or wavelengths of light.

The initial experimental hypotheses proposed that dietary zinc levels might influence light-induced retinal damage. Unfortunately, in most cases, the light stressed animals did not have statistically significant or clinically apparent signs of zinc deficiency. In the supplemented group only a few animals showed signs of a positive zinc status. Thus conclusions regarding the effect of dietary zinc level or zinc status on light damage cannot be made. Actually results from this study yielded contradictory conclusions. Subjective morphologic evaluations demonstrated the zinc normal and supplemented animals had more severe damage than those fed the zinc deficient diet. When kidney MT levels were used as an indicator of zinc status however, there was a trend, though non-significant, of the eyes of animals with lower kidney MT levels having a larger percentage of dying nuclei (Figure 47).

Melanin

One the most interesting findings in this study is that the light stress in the young porcine eye induced melanogenesis in the RPE and to a much lesser extent in the choroidal melanocytes. In many light-stressed porcine eyes all stages of melanogenesis were observed. The occasional developing melanosome was also observed in control eyes in all diet groups which shows that a low level of melanogenesis is still occurring in 6-8 week old pigs. Until recently the general consensus was that melanogenesis ceases before or shortly after birth. Studies on mammalian RPE cells have only revealed active formation of melanin granules during the fetal or perinatal period.[208] However, more recent investigations have found that some melanin turnover occurs in the eyes of adult vertebrates. Feeney in 1978 suggested that melanin degradation may be occurring in adult human eyes.[88] Using enzyme cytochemical studies she found that some melanin granules possessed hydrolytic enzymes. She described two types of melanin-containing complex granules, melanolysosomes and melanolipofuscin, and suggested that melanin commonly becomes incorporated into the lysosomal system of the RPE cell where it must undergoes modification and degradation. Additional studies substantiate her theory as they have shown that RPE and choroidal melanin content decrease with age in both white and black humans.[120] An inverse relationship between lipofuscin content and melanin in the RPE occurs with agemelanin decreases and lipofuscin increases. Using morphological and optical studies (fluorescence spectroscopy and microscopy) of melanin, Boulton et al. found an overall increase in density of melanin granules with age which they propose indicates melanogenesis is occurring within the human RPE throughout life.[209] Their study showed that existing melanosomes were becoming more optically dense presumably due to continuous deposition of the melanin polymer. It did not document the formation of new melanosomes, however, as was found here and in other studies. For example in porcine RPE fourth passage cell cultures, melanogenesis was still observed.[210] Melanogenesis has also been observed in cultured human RPE cells.[211]

Schraermeyer exposed adult hamsters to intense light (1000 lux) for 12 hours with the desire to enhance outer segment disc turnover and found melanogenesis in the RPE and to a lesser extent the choroid.[183,211] In the eyes that were exposed to the intense light, premelanosomes, and early and late stage melanosomes were all present.[211] Early stage melanosomes were also frequently associated with phagosomes, and some phagosomes contained a melanin-like compound. The author suggested that melanogenesis might be occurring in phagosomes as well as within the premelanosome that is synthesized by the endoplasmic reticulum.[183,211] Regardless, in the light-stressed adult hamster RPE and to a lesser extent the choroid, melanogenesis occurred. Large spherical late stage melanosomes with distinguishable melanofilaments (stage III melanosomes), ultrastructurally identical to those seen in porcine eyes in this study, were sometimes observed in the hamster RPE.[183] The author stated that the nature and origin of these bodies is unclear, but noted their size is similar to the length of normal adult RPE elliptical or lancet shaped granules. However, ultrastructural photographs of the human fetal RPE with active melanogenesis revealed the presence of similar large spherical stage III melanosomes as seen in this study and the hamster RPE.[184] Large spherical granules were also found in porcine RPE cell cultures.[210] These large granules may represent a normal development stage during melanosome synthesis. If during maturation of the granule, remodeling to a more elliptical shape did not occur, such a large spherical granule would represent an aberrant macromelanosome. Macromelanosomes, however, were not observed in any of the porcine eyes in this study.

In one instance it appeared as if melanogenesis might have been induced in the choroid of the light stressed porcine eye because melanofilaments were observed in some of the choroidal melanin granules. In the light-exposed hamster choroid, similar changes were observed ultrastructurally.[183] The author proposed that these changes may represent melanogenesis, or possibly melanin degradation, since melanin breakdown occurs in an order precisely the reverse of synthesis. The choroidal melanin changes in porcine eyes were observed too infrequently to conclude which process was occurring.

In many of the light exposed eyes, however, the choroidal and RPE melanin granule became angular, fragmented in appearance and more translucent without the presence of melanofilaments. These types of changes were observed to a lesser extent in eyes of animals that were maintained for three weeks after light exposure. In the hamster choroid, Schraermeyer described electron lucent spaces surrounding globular melanin subunits of different size and also loss of the normal round shape of granules with development of a more angular shape.[183] Fragmentation and translucent areas in RPE melanin granules of monkey eyes treated with argon laser has also been reported.[212] This type of change in the ultrastructural appearance of melanin granules may represent a light "bleached" melanin granule which may or may not subsequently undergo degradation. It appears however that light exposure activated melanin granules in the choroid and RPE of porcine eyes, because melanogenesis was occurring and increased degradation of melanin, i.e., as melanolysomes was also noted.

In the porcine eyes studied here, it appeared as if the dietary zinc level might be influencing the degree of melanogenesis. Subjectively, the animals on the Zn- and Zn+ diets had greater melanogenesis than animals fed the ZnN diet, with the eyes of animals fed the Zn- diets possessing the greatest amounts of melanogenesis. The propensity for the eyes of animals on the Zn- diets having the most melanogenesis was seen in the control group and in the continuous light group at 3 days and 3 weeks post-exposure. It was notable to observe melanogenesis in the eyes of animals 3 weeks post-exposure because these animals were 12-14 weeks old, an age at which melanogenesis would not normally be expected to be found. In light stressed animals maintained for 3 weeks after exposure, melanogenesis was found in both eyes of the animal on the Zn- diet, being very abundant in one eye, in one eye of the animal on the Zn+ diet, and in neither eye of the animal on the ZnN diet. The control animals on the diets for 3 weeks, and exposed to control lighting for the entire duration of the experiement, had less abundant melanogenesis, if observed at all. Melanogenesis was not observed in the either eye of the animal on the ZnN diet, was observed infrequently in one eye of the animal on the Zn+ diet, and was seen in both eyes of the animal on the Zn- diet. It is apparent that feeding the Zn- diet somehow induced or perpetuated perinatal melanogenesis and the addition of light stress markedly increased this affect.

The finding of low and high dietary zinc level stimulating melanogenesis must be substantiated by additional studies. Samuelson et al. did observe an increase in melanin surface area within the RPE in pigs fed a low zinc diet for several months.[21] Specifically, in control animals melanin occupied an average of 12.5% of the RPE layer suface area and in the low zinc group this increased to 17.9% of the cell area. The increase was not significant due to the large standarad deviation for melanin in the low zinc group RPE layers. Samuelson also observed a significant increase in RPE cell height and size in animals on the low zinc diet. This was attributed to increased abundance of residual bodies, melanin granules and phagosomes in the low zinc group. Despite an increase in melanin content, developing melanin granules with discernible melanofilaments were not observed. This contrasts with the frequent observation of immature granules in this study which probably relates to the age of the animals and the influence of the light stress. Sameulson's study utilized a longer experimental period as weanling pigs were acquired and fed a low zinc diet for several months. Depending on the experimental group, when eyes were removed for examination, animals were 5-13 month old animals. It may be that the increased RPE melanin content found in eyes of animals fed low zinc diets in Samuelson's study was the end product of stimulation melanogenesis at a younger age.

It was not surprising that increased dietary zinc might promote melanogenesis as zinc ions may catalyze some of the steps in the biosynthesis of melanin.[184] How low zinc status may promote melanogenesis is not clear. However, copper as well as zinc also can combine with dopa derivatives to form complexes that undergo nonenzymatic oxidation to form intermediates in melanin synthesis.[184] It is not uncommon for copper to increase as zinc decreases in tissues and this may help explain how a low zinc diet may promote melanogenesis.

Metallothionein

Metallothionein was found in very small quantities in the neural retina of the porcine eye. For control animals, the MT levels in the eye for each diet group were Zn- .02, ZnN 0.06, and Zn+ .02 µg MT/ mg protein. For the intermittent light group, the mean eye MT levels for the diet groups were Zn- .03, ZnN.04, and Zn+ .06 µg MT/ mg protein. The mean eye MT levels for the continuous light-exposed diet groups were Zn- .04, ZnN .05, and Zn+ .04 µg MT/ mg protein. There was no significant effect of dietary zinc on the MT levels in the neural retina regardless of light-exposure group (intermittent vs. continuous). The lack of effect of dietary zinc levels on ocular MT is not surprising, since there was no significant effect of the various diets on the zinc status of the animals. As mentioned previously, further dietary studies need to be performed in which diets are fed for longer periods of time and a significant alteration in zinc status is observed.

If diet is not considered as a variable, and the eye MT levels of each exposure group are compared, i.e., unexposed, intermittent and continuous, there is a trend toward increased eye MT levels in animals exposed to light vs. those not exposed to light. This suggests that light exposure may have induced MT synthesis in the neural retina. This is not surprising since MT seems to be a protein that is induced in periods of stress, particularly oxidative stress.[8,182] For example, in rats, glucocorticoids, glucagon, heat burn, hot environmental temperatures, exercise and CCl_4 have been found to induce liver MT synthesis.[8,158,182] Epinephrine and cAMP also increases cellular MT levels.[162] Studies with UV light found that MT increases as a result of UV light exposure.[8] Induction of MT occurs due to synthesis of the protein subsequent to activation of the MT gene. If light does induce MT, it must do so by generating the release of some substance that results in the MT gene activation. Further studies are needed to document whether or not light stress increases MT levels in the retina, and if it does, what types of gene activators mediate the induction.

The amount of MT found in neural retina, 0.02 - 0.05 μg MT per mg protein, is much smaller than that found in the retinal pigment epithelium. The assay was run on the wet tissue. The neural retina in both rats and rabbits has a high water content of around 83%. [213,214] The porcine neural retina probably has a similar water content so the amount of MT in this tissue may actually be more significant when considered on a dry weight basis. Cultured RPE cells have 7.6 μg MT / mg protein.[12] Induction of MT occurred after incubation with zinc, cadmium and dexamethasone to levels of 35.6, 55.2, and 19.2 μg MT/ mg protein, respectively. In another study of human RPE harvested from post-mortem eyes, macular RPE contained 17.6 μg MT/ mg protein in eyes from humans younger than 70 years, and 5.6 μg MT / mg protein in those over 70 years.[19] It would have been very interesting to determine how light affected MT levels in the RPE in this study, but this was not possible due to the choroid and RPE being sampled together, and the inability to analyze the choroid-RPE tissue. The small amount of tissue and the difficulty of getting the collagenous choroidal tissue to homogenize without losing significant amounts prevented quantification of MT on the choroid-RPE tissue.

It is not surprising that the RPE possesses a greater amount of MT than the neural retina, since MT is often abundant in epithelial tissue such as the intestinal mucosa.[8] The neural retina contains the photoreceptors, inner nuclear layer (Müller cells, bipolar, amacrine, and horizontal cells), and the ganglion cells and their axons in the innermost layers. It is likely the MT in the neural retina resides in the Müller cells, as they are glial cells with numerous metabolic activities that support the neural cells of the retina. Also, in the brain and spinal cord MT has been found in the glial cells, specifically, astrocytes.[215] Immunohistochemical studies on the rat retina showed that MT was present in the RPE and the inner retinal layers where Müller cells reside.[18] The investigators did not perform ultrastructural studies to localize MT on a cellular level, but the results obtained substantiate the presence of MT in the neural retina. Further studies utilizing immunostaining with ultrastructural examination would be useful to characterize which cells express MT and which cells respond to light stress or changes in zinc status by increasing the expression of MT.

Despite practicing the assay on tissues from slaughterhouse pigs before utilizing the experimental tissue, when the assay was run on the experimental animal tissues there may have been some inaccuracy introduced due to a slightly smaller amount of tissue in the samples. When developing the assay, Eaton and Toal performed studies to determine the accuracy of the assay.[181] They found that the assay was most accurate when the sample counts (minus background) were between 10 and 50% of the total counts. When sample counts were greater than 50% of total counts, the assay tends to underestimate the amount of MT. Dilution of the sample, so that counts are between the 10 and 50%, gives more reliable and consistent results. If sample counts are less than 10% of the total count the amount of MT may be underestimated. When the assay was run on the experimental tissues from the pigs in this study, several of the sample counts were less than 10% i.e. in the 5-7% range. Thus it is possible that the amount of MT in the porcine neural retina was underestimated. Future studies may require that groups of eyes be pooled to give a larger sample size. Also the sensitivity of the assay may be further increased by increasing the amount of radioactive cadmium.

This study and a few others have shown that MT is present in the retina and RPE of three different species: the rat RPE and inner retina , the pig neural retina, and in human RPE. [13,16,18] Another study identified a MT-like protein in the bovine OS.[11] Thus this ubiquitous protein is present in retinal tissues. It remains to be determined what role MT plays in normal retinal function and health. Metallothionein is a important protein in zinc metabolism and has been shown to play a significant role in regulation of zinc absorption from the intestinal lumen.[8,216] It may also facilitate or participate in zinc uptake from the serum by the RPE, which possesses tight junctions that represent the outer blood retinal barrier . Metallothionein also participates in the delivery of zinc to its apoenzymes, and in the retina it may direct zinc ions to melanin and/or metalloenzymes like superoxide dismutase and retinol dehydrogenase. In addition to its potential role in zinc metabolism within the retina, it may also provide an antioxidant function. Metallothionein has been shown to scavenge free radicals[8], such radicals are abundantly produced in the normal retina as it is exposed to light under conditions of high tissue oxygen content. Evidence of induction of RPE-MT by oxidative stress has been observed in several studies. For example, phagocytosis of latex beads or outer segments by the RPE, and exposure to hydrogen peroxideinduces MT synthesis.[13,16] Levels of transcription factors such as NF-kB and AP-2 which mediate the induction of MT transcription were also increased. Further studies will be required to better determine the role of MT in the normal retina and how this role changes with age, local factors such as light, and systemic factors such as poor nutrition in the elderly.

Elemental Analysis of Melanin Granules

The results of the x-ray microanalysis portion of this experiment did not show any significant effect of light and dietary levels of zinc on the melanin granule elemental content. The choroidal and RPE melanin x-ray spectra were significantly positively correlated and the correlation decreased in light stressed animals. This loss of correlation may be a manifestation of the light stress. The most abundant elements in the melanin granules of the choroid and RPE were sulfur, chloride, calcium, copper and zinc. Other studies have found similar content of ions in ocular melanin granules of pigs and humans.[147,151,217] Calcium was the most abundant cation in control animals and often in light treated porcine RPE and choroid. This substantiates the findings of other studies that suggested that pigment granules may be a calcium reservoir in the eye.[147,[illegible],218] Choroidal melanin granules had greater levels of calcium than RPE granules. This finding is similar to the study on adult sow utilizing x-ray microanalysis on porcine RPE and choroidal melanin.[151]

Iron was found in small amounts in the RPE melanin and rarely was seen in choroidal melanin granules. The only statistically significant effect of dietary zinc level on melanin granules was that low dietary zinc resulted in increased iron peaks in the RPE and choroid, although the amounts were still small. In another study on porcine ocular melanin granule ion content, iron was found to be more abundant in the choroidal granules compared to the RPE.[151] The differences between results of this study and the other study on porcine ocular melanin may relate to the light treatment of pigs in this study and the age of the pigs as the other study which utilized aged sows. Studies on human RPE melanin granules found higher levels of iron than in the porcine RPE granules in this study.[219] The human eyes were harvested from aged humans (> 65 years), and many had abnormalities such as basal laminar deposits so the differences compared to the porcine eyes studied here may relate to species, age and the presence of pathology associated with the RPE. It may be that with age the iron content of melanin granules in the RPE increases.

There was a tendency for the melanin granules of the light stressed group to have higher peak to background ratios of many elements, especially calcium, although this was not significant. One of the factors in the pathogenesis of light damage is membrane damage due to lipid peroxidation.[43,202] When membrane damage occurs, the ion content of the cells changes. Recently there has been much interest in elevated intracellular calcium as a major mechanism in cell death. This also is believed to occur in light damage as some investigators have shown that flunarazine, a calcium overload blocker, protected the rat retina against light damage.[220,221] Thus the melanin granules may act as a sink for calcium that is released into the cytosol from cell membranes during cell damage from light stress or some granules may respond to light by release of bound calcium ions. If melanin granule can act bind cytosolic calcium and bufferdevelopment of high intracellular calcium concentrations, this could represent another protective roll of melanin in addition to its light absorbing and antioxidant capabilities. The increase in iron seen in some eyes after light stress, although inconsistent, also could be protective against cellular damage because binding of iron to melanin would decrease free iron in the cytosol that might contribute to the Fenton reaction which may contribute to the oxidative damage induced by light.

It was notable that in some eyes exposed to light stress there were low levels of all elements. Perhaps a melanin granule with this type of spectra had been significantly damaged with a resultant loss of its bound metals and metal ion binding capacity. In several of our more severely damaged eyes, we observed fragmented melanin granules ultrastructurally, and in some cases melanolysosmes, suggesting turnover of melanin. Melanogenesis appeared to be occurring in several eyes of these young pigs, especially in the light treated group. It has been reported that immature melanosomes and premelanosomes in the skin contain greater amounts of metal ions than mature melanosomes.[184] It would be useful to be able to identify structurally altered melanin granules or immature melanosomes, and then perform x-ray analysis on such granules in order to try to correlate the elemental content of the granule with its ultrastructural appearance. Unfortunately this was not possible because the slam freezing technique does not preserve the melanin ultrastructural appearance well enough to consistently identify fragmented or immature melanosomes.

As mentioned previously, the dietary zinc level did not appear to influence the melanin ion content significantly. However in some animals on the low zinc diet the melanin granules had very low content of all ions compared to controls. Another study on porcine eyes examined the effects of chronically feeding intermediate and low levels of dietary zinc on aged porcine eyes.[151] In that study the low zinc levels appeared to decrease zinc and iron while copper increased. Those findings differ from this study in which iron appeared to increase as a result of feeding the low zinc diet. In this study however, light treatment may have contributed to the difference in the elemental content of melanin granules. In addition the diets were fed for a shorter period of time and also the animals were much younger.

There were several problems encountered utilizing elemental analysis to investigate the effects of light damage. We chose the slam freezing technique to analyze the ocular melanin since this method avoids loss of elements in tissues that can occur with fixatives, and alcohol treatments that are used in routine embedding in plastics.[178] Sodium and potassium are two elements that have been shown to disappear if regular fixation and embedding techniques are utilized.[178,222] The slam freezing technique, however, results in distortion of the RPE cell layer so that it is hard to tell if the melanin granule is in a normal or damaged cell. This makes it difficult to correlate changes in spectra of specific melanosomes with cellular damage. Also, since our light damage was probably very near threshold, damage was multifocal and often mild. It is conceivable that many areas that were examined by x-ray microanalysis could have been normal, and thus did not reflect a change due to feeding the different experimental diets and/or lighting conditions.

From this study we have learned that calcium, copper, zinc and iron are main elements found in spectra other than sulfur, chloride, potassium and sodium. Calcium, iron, copper and zinc are important ions in RPE and melanin granules. We have analyzed melanin granules from slam frozen and glutaraldehyde fixed tissue, and compared the spectra.. The calcium, iron, copper and zinc peaks do not significantly differ when comparing frozen vs. glutaraldehyde fixed tissues that are both processed rapidly. Subsequent studies may utilize tissue fixed for morphological examination so that damaged areas can be isolated and the melanin granules analyzed. Also this may allow identification of damaged or immature melanin granules for analysis. This will enable better interpretation of the effects of light on melanin ion content. A better alternative may be to develop further, the light damage model in the pig to the point where diffuse and significant damage can be consistently reproduced. The slam freezing technique would then be more useful as it would provide more reliable information.

Finally, it deserves comment that the literature in many cases mentions the difficulty of utilizing x-ray microanalysis techniques in biological tissues.[178,179] It is very difficult to use this tool quantitatively because of the variability of spectra peak areas. The area of elemental peaks can be influenced by several variables, such as section thickness, accelerating voltage of the electron microscope which can vary from day to day, or from the fixation method and processing techniques. We found that variability in the scope parameters from day to day can be minimized by utilizing a standard. If RPE granules from a selected section that is used as a standard are analyzed each day at the beginning and end of the scope session, spectral peak to background ratios can be reliably compared. If the standard spectra are similar from day to day it suggests it is valid to compare spectra generated from samples. If the standard differs significantly, the sample spectra may be adjusted accordingly. The use of this kind of method obviously does not allow quantitation of exact quantities of elements, but it does allow qualitative comparison between samples, and allows observation of significant alteration of peak to background ratios as effect of variables such as age, diet and light.

Recently we have found that the melanin granules may retain some of its ability to release and bind elements even while in plastic. Serendipitously, when sectioning with a new diamond knife, a prominent nickel peak appeared in the spectra obtained from a pig eye and an alligator eye that were sectioned on the same day. The nickel peak, however, did not appear in the cytosol or background spectra. Repeat sectioning on the same block with a different knife yielded x-ray spectra that lacked any nickel peak. Thus melanin appeared to bind nickel after fixation during the sectioning process. The manufacturer of the knife informed us that the diamond knife was secured to the knife holder with an adhesive that contained nickel. Thus it seems that either a nickel residue or the nickel from the adhesive dissolved into the distilled water solution upon which sections are floated during ultrathin sectioning. Subsequently, it has been found that if sections are floated on deionized water for 15 minutes, the elemental content changes with time, with several elements such as calcium, copper and zinc being either lost or gained depending on the floating solution.

Some of the unexpected results, i.e., in one eye a very large zinc peak was noted in the eye of an animal on the Zn- diet, may have been due to contamination of the floating solution, and also due to how long each section was floated on the solution. For example, some specimens from which unanticipated elemental spectra were generated were resectioned without floating on the water for more than 30 seconds and reanalyzed. The previous unexpected findings, i.e., the high zinc peak in an animal on the low zinc diet when resectioned was no longer present. It is likely that no significant trends were observed as a result of light because of the sectioning technique was not strictly standardized.

The discovery of the ability of melanin in cryopreserved tissue to uptake or lose elemental content depending on the ion content of the solution upon which sections are floated has led to a revision in our technique. Our technique has been revised and we now use deionized, filtered, high performance liquid chromatography water to float out sections. In addition sections are picked up within 30-45 seconds after floating on the water to limit any exchange between the water and the melanin granule. Studies are underway to better characterize how melanin granule spectra change with time on the floating solution, and when different concentrations of elements are within the floating solution. Preliminary results suggest dietary zinc level and light may influence the ability of ocular melanin to bind cations such as calcium and zinc.

Summary

The porcine retina was characterized morphologically by light and electron microscopy and found to possess a high number of cones that are concentrated in an area in the superotemporal fundus, the area centralis. The area centralis of the porcine eye appears to be horizontally oval, rather than the distinct round macula that is found in primates.

High intensity light was delivered to free-ranging pigs utilizing different schedules of intermittent light (36 or 72 hours with a 18 hour light and 6 hour dark period) or for 72 continuous hours. Two animals received the 36 and 72 hours of intermittent light under conditions of hyperthermia. This study found that light stress induced retinal damage in the porcine eye. Two types of damage were observed. The first type was characterized by multifocal outer nuclear layer pyknosis, and with ONL nuclear movement outward through the external limiting membrane. This damage appeared to be most prevalent in the intermittent light group. A second more severe type of damage, resembling Noell's type II damage of rats, was found in the light stressed porcine eyes. This damage was rarely observed in the intermittent light stress group, was seen in animals exposed to intermittent light under conditions of hyperthermia and also observed in the continuous light group. This damage presumably represents an acute and more severe type of damage. It is characterized by diffuse retinal edema, diffuse outer nuclear layer pyknosis or chromatolysis , occasionally inner nuclear layer cell death or swelling, OS and IS edema and degenration, and RPE edema, necrosis and detachment from Bruch's membrane. The light-damaged porcine eye represents a good model to study the effects of light on the eye, and will be particularly useful as a model for primates because it possesses a high concentration of cone photoreceptors. The pathology of the light damage induced in the porcine eye is remarkably similar to that seen in primate studies. In addition, as has been shown in other studies, heat stress accentuated the light-induced retinal damage. The light damage in this study in most cases was not severe, resulting in scattered damage, which led to the conclusion that the light dose was near the threshold for the porcine retina. This was further substantiated by the fact that at three weeks post-exposure the retinas had morphologically recovered with only some signs of previous damage.

Different experimental diets with low, normal and high levels of Zn were fed to light exposed animals to determine any influence of diet on light damage. The zinc status of the animals was not significantly altered so interpretation of any affect of zinc status on the photic injury could not be performed. In animals with lower kidney MT levels, more severe damage and increased percentage of dying nuclei were observed. This suggests that a subclinical zinc deficiency may have accentuated the photic injury to some degree, but future studies are needed to confirm this finding.

This study revealed that melanogenesis is occurring to a minor degree in the eye of 6-8 week old pigs and substantiated the emerging theory that melanogenesis does not cease at birth or in the early perinatal period as has been previously believed. Feeding the low and high dietary zinc levels appeared to increase mildly melanogenesis in the young porcine eye. Light stress markedly increased melanogenesis in the young porcine eye. There appeared to be a synergistic positive influence of light and dietary zinc. High and to a greater extent, low dietary zinc, subjectively appeared to induce the most melanogenesis. In addition, choroidal melanogenesis was seen in one eye so the light may also be influencing choroidal melanin dynamics. In the choroid and RPE of light stressed eyes melanolysosmes were sometimes observed. This finding indicates melanin degradation was also occurring. Thus light and dietary zinc level appear to alter melanin dynamics in the young porcine eye. The light-stressed pig eye may represent a good model for studying melanogenesis and factors that influence it.

The cadmium hemoglobin binding assay was utilized to determine the amount of MT in the porcine neural retina. The neural retina of control animals contained an average of

0.03 µg MT / mg protein. The amount of MT in the neural retina did not appear to be significantly altered by diet or light. Light exposure however showed a tendency towards increasing the retinal MT level. This response needs to be confirmed in further studies but would not be surprising, as MT is a protein that is induced by several factors and stresses.

The goal of this study was to utilize the pig as a model to study two pathogenetic factors of ARMD in humans, poor zinc nutritional status and cumulative light exposure. Therefore, the experiment was designed to mimic the type of exposure that humans might receive during their lifetime, i.e., rather than extremely high intensity light exposure over a short time period, chronic relatively high exposure might occur. Also, marked and acute zinc deficiency does not commonly occur in the normal human population. Rather, the elderly human population suffers from subclinical mineral and vitamin deficiencies. Therefore, the experimental design utilized free ranging pigs being exposed to moderately intense light for several days. The one factor that this study did not include in the design of this study was the influence of age, as all our experimental animals were a few months old. Also our light exposure regime was not chronic in nature in that only 72 hours of cumulative light exposure was performed. These factors, in part may explain why severe retinal damage was not induced by the light stress in the experimental animals. The cost of performing longer term studies prohibited increasing the length of the experimental period in this study. However, this pilot study investigating the effects of dietary zinc level on light stress to the retina of the pig has generated interesting results that will allow more effective experiments to be designed in the future.

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BIOGRAPHICAL SKETCH

I received a Bachelor of Science degree in zoology at the University of Florida in 1984. Subsequently, I enrolled in graduate school at the University of Florida and pursued a Master of Science degree in comparative ophthalmology, in the College of Veterinary Medicine. After receiving the M.S. degree, I entered veterinary school at the University of Tennessee College of Veterinary Medicine. I received my Doctor of Veterinary Medicine degree in 1988. From 1988 until 1989, I continued my clinical education at North Carolina State University College of Veterinary Medicine, as a small animal intern. In 1989, I returned to the University of Florida as a Ph.D. graduate student in the College of Veterinary Medicine. Again my studies were in the field of comparative ophthalmology. In 1990, I was accepted as a resident in comparative ophthalmology in the Department of Small Animal Clinical Sciences, College of Veterinary Medicine. From 1990-1993 I was both a resident and graduate student. I completed my residency and successfully achieved board certification, becoming a Diplomate of the American College of Veterinary Ophthalmologists in 1993. I then accepted a position as a Provisional Assistant Professor with the Department of Small Animal Clinical Sciences, University of Florida College of Veterinary Medicine, and completed my Ph.D. degree during the period from 1993 until 1995.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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